USE OF VEGETABLE OIL IN REDUCTIVE DECHLORINATION OF TETRACHLOROETHENE

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by

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ABSTRACT

Reductive dechlorination is an effective bioremediation method for treating tetrachloroethene and its daughter compounds. Common techniques of stimulating reductive dechlorination involve the injection of costly, soluble electron donors into the contaminated plume. Vegetable oil is a cheaper alternative to such donors on a cost-per-mass basis, and may even be more economically implemented. This study applied microcosm studies to investigate the effectiveness of vegetable oil as an electron donor. Cultures fed with vegetable oil were observed to completely dechlorinate tetrachloroethene to ethene. Dechlorination by vegoil was also sustainable over a period of 140 days without the addition of nutrient amendments. Nevertheless, vegetable oil was found to ferment relatively quickly, leading to low donor efficiency.

Biomass and acetate were the most significant products of vegoil-fed microcosms. Volatile fatty acids longer than 2 carbons rarely persisted. Since these acids can act as good secondary donors in the aqueous phase, their absence implies that the dechlorination zone does not extend very far from the vegoil phase. Inference from biomass measurements and dechlorination behavior hints that endogenous decay of

large quantities of biomass could provide a stable source of electron donor. Perhaps a similar method of growing up a large pool of biomass for electron-donating purposes could be investigated in the future.

Since acetate rarely ferments further to produce more hydrogen, and methanogenic biomass is fairly immobile, one good area of application for vegoil would be in bio-barriers. Interception of a contaminated plume by constructing biobarriers downstream reduces the need to maintain a large zone of treatment, although treatment times could be longer. For example, vegetable-oil-coated sand particles could be used to back-fill a trench to intercept and treat a plume consisting of dissolved chlorinated ethenes.

BIOGRAPHICAL SKETCH

Sin Chit To, or better known as Ernest, was born in July 27, 1975 in Hong Kong. He spent the earlier part of his childhood in Singapore while his formative years were in Hong Kong. He came to the United States in 1993 to pursue a degree in the University of Texas at Austin in the field of Civil Engineering. Following his graduation in 1996 he left for Singapore to fulfill two and a half years of compulsory military service. He served as an officer in an infantry battalion. In 1999, he enrolled in Cornell University to study for a Master of Science Degree in Environmental Engineering. He now works in New Jersey.

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TABLE OFABBREVIATIONS

CH_4	methane
ETH	ethene
1,1-DCE	1,1-dichloroethene
cis-1,2-DCE	cis-1,2-dichloroethene
cDCE	cis-1,2-dichloroethene
DNAPL	dense non-aqueous phase liquid
FID	flame ionization detector
GC	gas chromatography
H_2	hydrogen
LNAPL	light non-aqueous phase liquid
NAPL	non-aqueous phase liquid
PCE	tetrachloroethene, perchloroethylene
PON	particulate organic nitrogen
RGD	reduction gas detector
TCE	trichloroethene
TKN	total Kjeldahl nitrogen
VC	vinyl chloride

VFAs	volatile fatty acids
VSS	volatile suspended solids

CHAPTER ONE

INTRODUCTION

1.A. Context

1.A.1 Environmental Problems Associated with Groundwater Contamination by Chlorinated Solvents

1.A.1.a. Extent of Pollution caused by Chlorinated Solvents in the US. Tetrachloroethene (perchloroethylene, PCE) and its daughter compounds –trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) in groundwater pose a complex and persistent threat to the environment. Groundwater contamination has been associated with 300,000 to 400,000 hazardous waste sites in the United States. PCE and TCE are among the most frequently detected contaminants in groundwater. TCE and PCE are ranked as the first and third most-frequently detected pollutants at hazardous waste sites [*39*]. Available data from the 1430 sites found on the National Priority List (as identified

by the USEPA) in 1997 show that PCE and other chlorinated ethenes were present in at least 771 sites [*2*]

Until recently, PCE and TCE were manufactured extensively because of their wide application as organic solvents in degreasing and dry-cleaning purposes. They are nonflammable, thus safer in that respect for workers to handle than some alternatives, such as petroleum distillates. The use of TCE in the military as an engine-degreaser, and PCE primarily in the domestic dry-cleaning industry and in CFC production, increased rapidly in the 1960s [*8*]. Their use leveled out in the 1980s, but spills and improper disposal of these DNAPLS (dense non-aqueous phase liquids) at hazardous waste disposal sites, domestic dry-cleaning establishments, military bases, and industrial complexes has resulted in many contaminant plumes. Nowadays, the use of chloroethenes is decreasing because of regulatory changes intended to phase out their use due to their carcinogenic or potentially carcinogenic nature [*60*].

1.A.1.b. Regulatory Standards for Chlorinated Solvents. The Department of Health and Human Services (DHHS) has determined that PCE and TCE may reasonably be anticipated to be carcinogens. [*2*]. PCE has been shown to cause liver tumors in mice and kidney tumors in male

rats, although its effects on humans are still unknown. The EPA maximum contaminant level for both PCE and TCE in drinking water is 0.005 mg/L. For DCE (including 1,1-DCE, cis-1,2-DCE and trans-1,2-DCE), the long-term human health effects following exposure to low concentrations are still unknown. Nevertheless, the EPA has ruled that the highest level of DCE in drinking water be less than 7 μg/L and any release of more than 5,000 lb to the environment be reported. Unlike PCE, TCE and DCE, VC has been classified by the Department of Health and Human Services as a known human carcinogen [*2*]. VC exposure causes liver cancer in people [*2*]. The EPA requires that the amount of vinyl chloride in drinking water not exceed 0.002 mg/L and requires that spills or accidental releases into the environment of 1 pound or more of VC be reported to the EPA [*2*].

1.A.1.c. Characteristics of Groundwater Contamination by Chlorinated Solvents. PCE and TCE exist at room temperature as liquids denser than water (specific gravity of PCE and TCE are 1.62 and 1.46 respectively). [*34,35*]. Therefore, when PCE and TCE are leaked into the ground, they tend to migrate downwards to the base of aquifers and form globules of DNAPL (dense non-aqueous phase liquid) that are

difficult to remove, detect or trace. A tiny but significant portion dissolves into the groundwater forming an aqueous contaminant plume that is carried downgradient from the source (see Figure 1.1). PCE and TCE contamination are also often associated with mixed organic non-aqueous phase liquids because of their limited aqueous solubility and high miscibility in other organic solvents.Source zones, or areas in which NAPLs are present, represent long-term sources of groundwater contamination [*42*], and their presence greatly complicates the ability to restore contaminated aquifers.

1.A.2. Conventional Methods of Treating Groundwater Contaminated by Chlorinated Solvents

Finding practical methods to remove chlorinated solvents and other contaminants from groundwater has always been a major challenge to environmental professionals. The conventional way of treating groundwater contaminated by chloroethene used to be “pump-and-treat” which is the pumping of contaminated water to the surface for treatment. Above-ground treatment usually takes the form of physical means, such as air-stripping or carbon adsorption, in which the pollutant is transferred

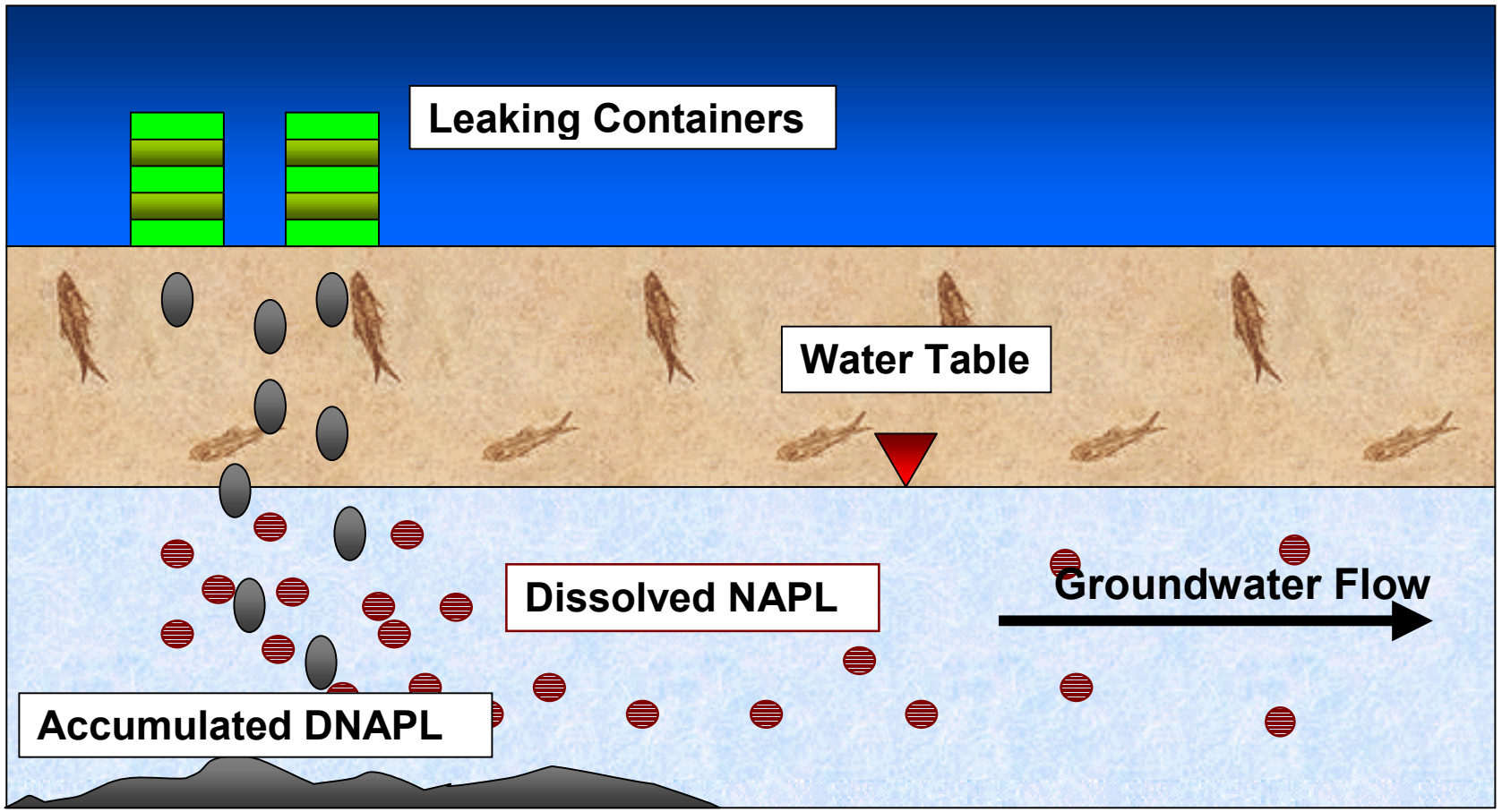

Figure 1.1 Contamination by Chlorinated Solvents in the Field

from one phase to another. Since the early 90's, this sort of "*ex-situ*" treatment has been recognized as an ineffective method when used alone for source zone restoration [*38*]. Therefore more attention is now directed towards biological/chemical process that perform treatment under the subsurface and inside the contaminant plume. This is known as *in-situ* bioremediation.

1.A.2.a. In-Situ Treatment. *In situ* bioremediation (ISB) is the use of microorganisms to degrade contaminants in place with the goal of obtaining harmless chemicals as end products. Most often *in-situ* bioremediation is applied to the degradation of contaminants in saturated soils and groundwater, although bioremediation in the unsaturated zone can occur. The technology was developed as a less costly, more effective alternative to the standard pump-and-treat methods used to clean up aquifers and soils contaminated with chlorinated solvents, fuel hydrocarbons, explosives, nitrates, and toxic metals [*4*].

Enhanced *in-situ* bioremediation is where substrate and/or nutrients are added to an aquifer to stimulate the growth of a targeted consortium of bacteria. Usually the targeted bacteria are indigenous, however enriched cultures of bacteria (from other sites) that are highly efficient at degrading

a particular contaminant can be introduced into the aquifer (termed bioaugmentation). Chlorinated ethenes often require the addition of an electron donor to stimulate reductive dechlorination. The goal of enhanced *in-situ* bioremediation is to increase the targeted biological activity biomass throughout the contaminated volume of aquifer, thereby achieving effective biodegradation of dissolved and sorbed contaminant.

1.A.3. Reductive Dechlorination

Reductive dechlorination is the process in which chlorine atoms are replaced with hydrogen atoms in a stepwise process (see Figure 1.2). Under anaerobic conditions, PCE is reductively dechlorinated to its lesser or non-chlorinated daughter compounds TCE, DCE isomers, VC and ETH by a number of organisms. Molecular hydrogen (H_2) has been identified as the reductant; as such the reaction is very energetic. The anaerobic process becomes slower as the number of chlorines decreases. However, TCE, DCE, and VC are degradable aerobically via co-metabolic activities, and the efficiency of aerobic treatment generally increases with a decreased number of chlorines.

Reductive dechlorination has been demonstrated under nitrate- and iron-reducing conditions, but the most rapid biodegradation rates,

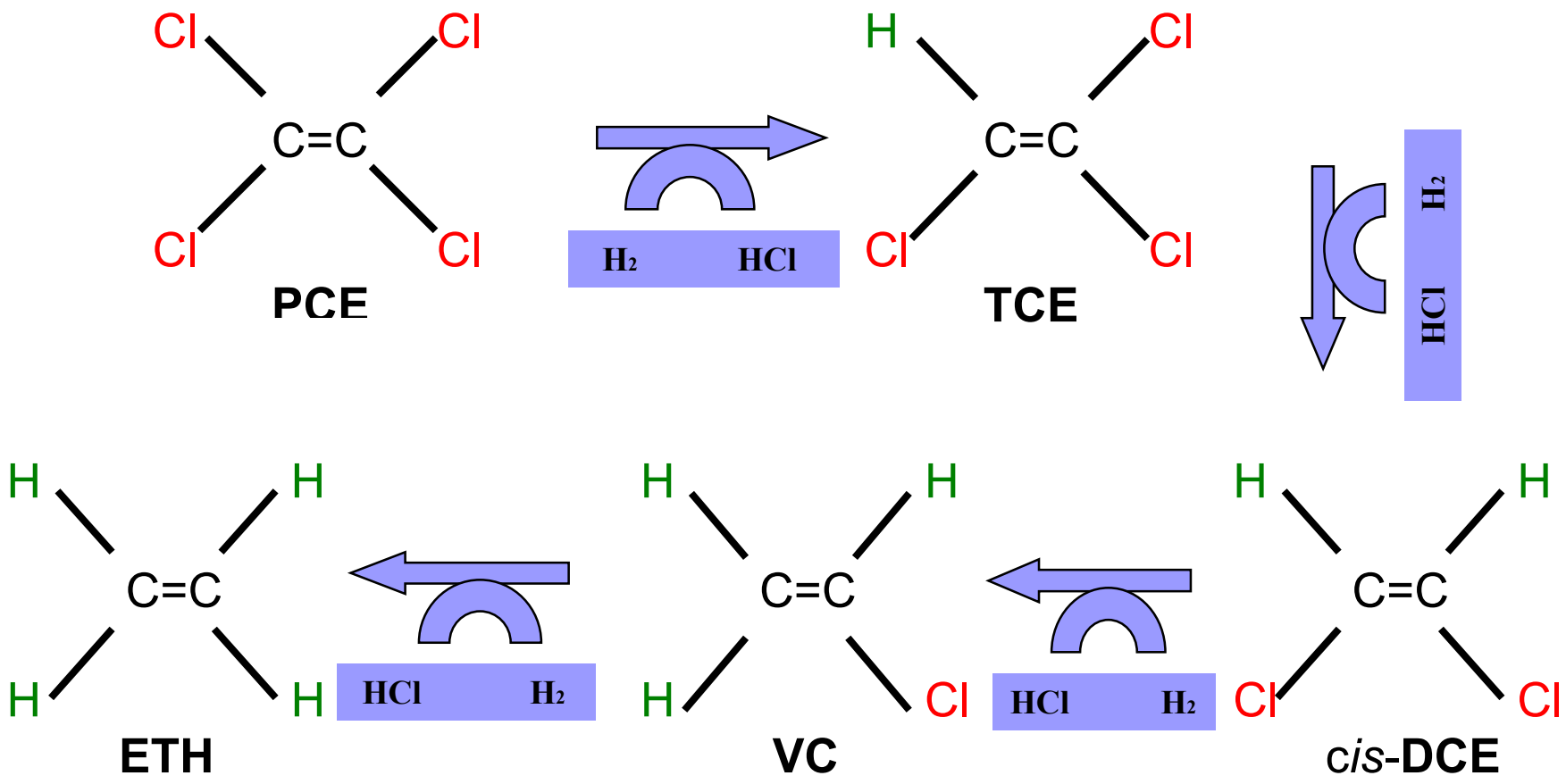

Figure 1.2. Reductive Dechlorination Pathway

affecting the widest range of chlorinated aliphatic hydrocarbons, occur under sulfate-reducing and methanogenic conditions. [*4*]. Reductive dechlorination requires an external electron donor that produces H_2. H_2 can be produced in large quantities through direct fermentation of substrates such as complex organic compounds and volatile fatty acids such as butyrate; or as a trace intermediate as in the conversion of methanol to acetate, or even in the conversion of methanol or acetate to CH_4.

While VC is believed to be the most harmful compound of the series, its production under anaerobic condition is no longer thought to be disastrous since it is known to be readily oxidized under aerobic conditions by isolates that use it as a primary substrate [*26*], environmentally-occurring organisms [*12*], methanotrophic cultures [*40*], ethane- and ethene-degrading cultures [*22,23*], and iron-reducing organisms [*7*]. At many sites, if dechlorination can be pushed to VC in the anaerobic zone, then the residual VC is readily degraded as the plume converts to aerobic conditions. If however, DCE is the final product of the anaerobic zone, it may persist in these aerobic zones.

1.A.4. Stimulation of Reductive Dechlorination using Vegoil as Electron Donor

The vegoil process was proposed by Parsons Engineering Science, Inc. as a novel approach for stimulating reductive dechlorination of chlorinated solvents. It is essentially the addition of vegetable oil (hereby abbreviated as "vegoil") as a substrate to generate H_2 and stimulate dechlorination. It is potentially cost-effective approach for the remediation of contaminants that are anaerobically biodegradable. Such compounds include the chlorinated ethenes, ethanes, methanes, and benzenes.

Vegoil is a triacyglycerol consisting of long-chain fatty acids with lengths of 8 to 18 carbons. It is an inexpensive, innocuous, food-grade carbon source that is not regulated as a contaminant by the EPA. Because vegetable oil is a NAPL, the potential exists that a single, low cost, injection could provide sufficient carbon to drive reductive dechlorination for many years. This will significantly lower operation and maintenance costs compared to aqueous phase injection, and will allow injection of a much greater quantity of carbon than solid phase carbon emplacement.

Vegetable oil is a versatile compound which can exist either as a liquid (such as common soybean oil), solid (such as palm kernel oil) or an

emulsified mixture (with the addition of an emulsifying agent, such as lecithin). As a liquid, significant amounts of vegetable oil can be injected directly into an aquifer utilizing conventional wells. It is projected that the separate-phase nature of vegetable oil would allow for slow dissolution into groundwater thus making it a slow release carbon and H_2 source.

The vegoil process could have the potential to be used either in the contaminant source zone, or down-gradient as a barrier to contaminant migration. In the barrier configuration the process could act similarly to iron filings barriers, but at a lower cost and possibly with fewer operational problems.

Previous reductive dechlorination laboratory studies that used other well-defined electron donors such as gaseous hydrogen, lactate and butyrate, etc. eventually failed over the long term when the addition of nutrients such as yeast extract and vitamin B_{12} was omitted. Thus if vegetable oil – a naturally-occurring mixture of compounds – itself contains such nutrients, or could form such nutrients from its breakdown process, the need for such additions could be eliminated. This could significantly simplify the implementation process.

1.A.4.a. Technology Maturity of Vegoil Process. The VegOil approach has been applied at a site at Cape Canaveral Air Station in Florida; it is scheduled for application at an Army site on the Defense Depot Hill Utah and Travis Air Force Base in California [*42*]. These ongoing applications are limited in scope and while they do serve to demonstrate the viability of the process additional work is required to raise the process to the level of a routinely available commercial process.

1.B. Experimental Strategy

1.B.1. Experimental Objectives

The overall objective of this study is to demonstrate in a laboratory setting that vegetable oil is a sufficient and adequate electron donor to drive anaerobic dechlorination. Although some field evidence of successful application of vegoil already exists [*42*], in order to advance into commercialization, meticulous microcosm studies would be required in order to better characterize vegoil-stimulated dechlorination. To be more specific, such microcosm studies would seek to answer the following questions:

1.B.1.a. Biologically/Reductive Dechlorination-Related Objectives

1. Does vegoil stimulate the complete dechlorination of PCE to ETH?
2. Does vegoil ferment slowly to produce a persistent pool of H_2 at low partial pressure so as to support more efficient dechlorination?
3. Does the breakdown or fermentation of vegoil produce any nutrients that may assume the role of yeast extract and vitamin B_{12}? On the other hand, does breakdown or fermentation of vegoil produce any compounds inhibitory to the dechlorinators?

1.B.1.b. Applications/ Engineering-Related Objectives

1. Does the low solubility of vegoil allow regulation of donor:PCE ratios at stoichiometric levels? (Regardless of whether donor is slowly or quickly fermented, excessively high donor:PCE levels will simply cause excess donor to be scavenged by methanogens, leading to low donor efficiency.) Self-regulation of low donor:PCE levels signifies that one

large donor injection can sustain dechlorination in a contaminated site for many years.

2. Does breakdown of vegoils produce any persistent long-chain fatty acids? Long-chain fatty acids soluble in water are hoped to be carried along with the contaminant plume and act as a persistent source of slow fermenting hydrogen. This consequently enlarges the treatment zone outwards from the vegoil layer.

3. How does vegoil compare in performance to (Hydrogen Release Compound) HRC® in terms of stimulating dechlorination? Vegoil and HRC® are both low-solubility donors that have similar chemical structure – HRC® is glycerol tripolylactate, while vegoil consists of triacylglycerols. Commercial literature claim that HRC® is a very effective hydrogen donor [*43*]. However, vegoil is a much cheaper alternative to HRC®.

1.B.2. Experimental Strategy

To achieve the above-mentioned objectives, microcosm studies were prepared to study the behavior of vegoil-fed, mixed-dechlorinating culture. An anaerobic mixed-culture, consisting principally of a well-characterized dechlorinator (*Dehalococcoides ethenogenes*, Strain 195), various fatty-acid fermenters, acetotrophic methanogens, and hydrogenotrophic methanogens was employed in this study. This culture had been steadily maintained for several years on a feed of PCE, butyrate, yeast extract, and a vitamin mixture containing B_{12}. Since this culture regularly received fatty acids (butyrate), it was projected that it could also utilize vegoil and its breakdown products.

Microcosm studies were prepared by anaerobically transferring 100 mL aliquots of the culture to 160-mL serum bottles, Three types of vegoil and HRC® were tested as substrates. Within each substrate sample, some bottles were fed with yeast extract and vitamin B_{12} and while others were not. This was to assess the availability of such nutrients based on the dechlorination performance. After setup, the following time-course profiles of the various bottles were monitored:

- H_2,

- Chloroethenes
- CH_4
- Volatile Fatty Acids (VFAs - acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic)

Unfed controls were prepared to allow the role of the oils to be assessed. The microcosm studies ran for four to five months.

Data obtained from the time-course profiles were used to answer the principal points of investigation in the following manner summarized by Tables 1.1 and 1.2.

Table 1.1 Biological/Reductive Dechlorination-Related Objectives

	Potential Advantages of Vegoil	**Method of Investigation**
1.	Able to sustain complete dechlorination from PCE to VC and ETH	Monitoring of chloroethene profiles of vegoil-fed bottles Check for accumulation of intermediary products, i.e. TCE and cis-DCE or cessation or decrease in dechlorinating activity
2.	Ferments slowly to support dechlorination	Monitoring of hydrogen profile of vegoil-fed bottles
3.	Contains nutrients that sustain long-term dechlorination Contains inhibitors that impede or even halt dechlorination process	Comparison of chloroethene profiles of vegoil-fed bottles amended with yeast extract and vitamins against those not amended Check for accumulation of intermediary products, i.e. TCE and cis-DCE or cessation or decrease in dechlorinating activity

Table 1.2 Application/Engineering-Related Objectives

	Potential Advantages of Vegoil	**Method of Investigation**
1.	Slow dissolution provides mechanism for maintaining low donor:PCE ratio in samples.	Inference from methane, hydrogen and profile of various vegoil-fed samples
2.	Breakdown of vegoil produces long-chain fatty acids	Monitoring of VFA production in samples Check for VFAs longer than 2 carbons (acetate)
3.	Performs better than same amount of HRC or performs better than same cost of HRC	Comparison of chloroethene, hydrogen and methane profiles of HRC-fed bottles against vegoil-fed bottles Check for rate of dechlorination and accumulation of intermediary products, i.e. TCE and cis-DCE

CHAPTER TWO

BACKGROUND

2. A. History

During the early 1980s, McCarty and co-workers performed studies on mixed cultures and bioreactors that were subjected to PCE and TCE inputs. Such studies showed the possibility of PCE and TCE being dechlorinated to the DCE isomers [*5,6,9*] and VC under anaerobic, methanogenic conditions [*56,57*].

In 1989, the first evidence that reductive dechlorination proceeded completely to the non-chlorinated, environmentally benign compound, ethene (ETH) was provided by Freedman and Gossett [*22*]. Freedman and Gossett used a sixth-generation anaerobic culture that was originally seeded with digested sludge from a local municipal wastewater treatment plant and then fed frequently with PCE- and TCE-saturated stock solutions. Complete dechlorination in the culture relied on the addition of large ratios of electron donor to PCE or TCE on an equivalents basis. Only a tiny fraction of the donor was channeled to dechlorination, but

since the amount of chloroethene added was small, dechlorination was complete.

DiStefano et al.[*14,15*] converted the mixed-anaerobic cultures of Freedman and Gossett to a non-methanogenic, primarily dechlorinating culture. This was achieved by incrementally increasing the PCE loading to the point where methanogenesis was largely inhibited. Methanol was used as an electron donor, and its principal fate was acetogenesis, under inhibited methanogenesis. In this way, a higher concentration and proportion of dechlorinators was cultivated, and isolation of the dechlorinator was possible. This culture is referred to as the high-PCE/methanol culture. As PCE dosage to the high-PCE/methanol culture increased from 250 μM to 550 μM, CH_4 production by acetotrophic and hydrogenotrophic methanogens declined and finally ceased. Direct methanogenesis from methanol was also inhibited by the PCE dosage. Apparently, high concentrations of PCE inhibited the competition of reducing equivalents by methanogens (see Figure 2.1).

When incubation was allowed to proceed for as long as 4 days, virtually complete conversion of PCE to ethene resulted, with less than 1% persisting as VC. An electron balance showed that 31 percent of the added methanol equivalents (based on oxidation to CO_2 basis) was

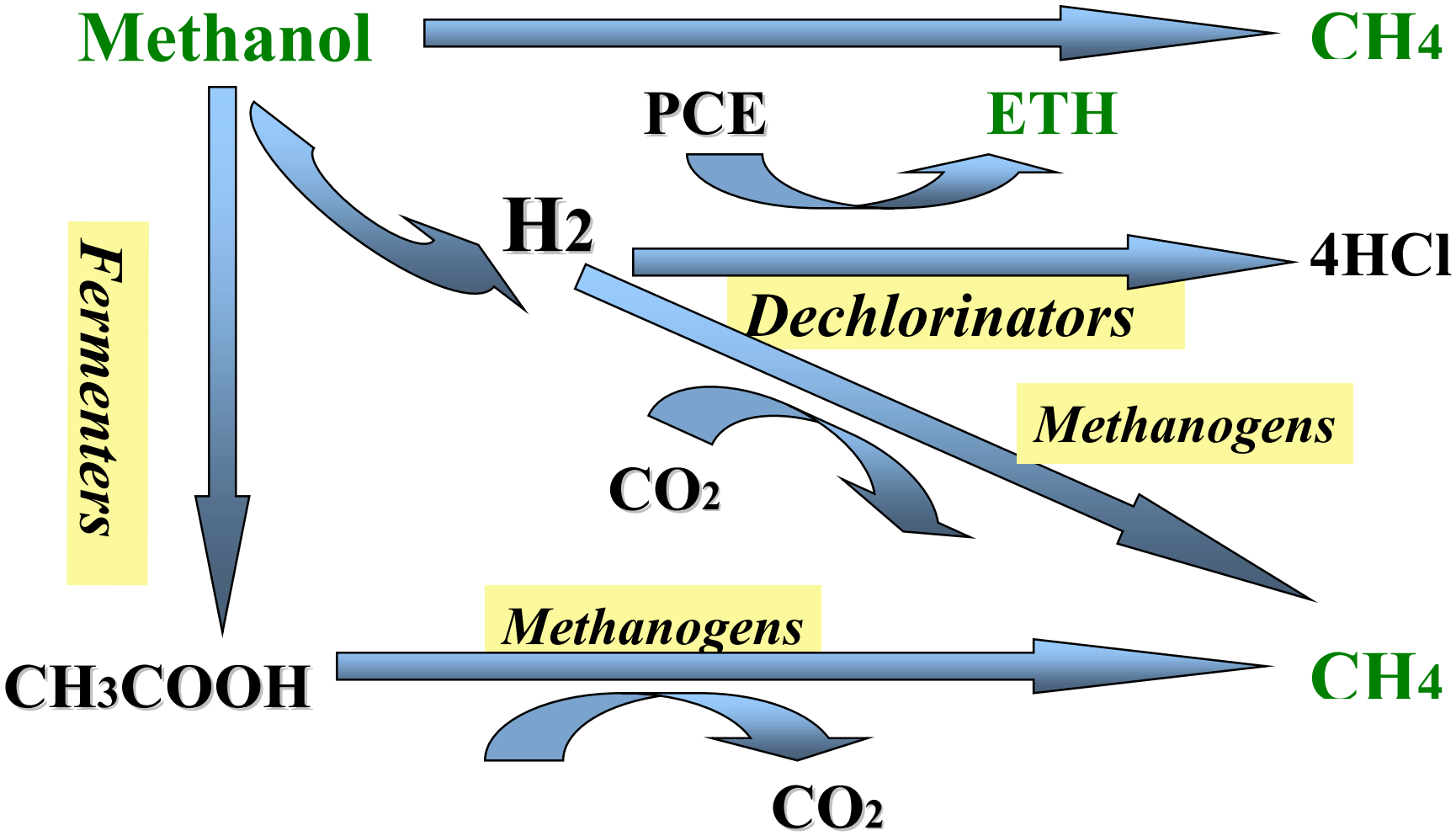

Figure 2.1 Interaction between Microbial Agents in Reductive Dechlorination using Methanol as Electron Donor (Non-Inhibitory PCE concentrations)

channeled to dechlorination and 69 percent was channeled to acetate production.

Further studies by DiStefano [*14,15*] confirmed that H_2 was the electron donor used directly by the dechlorinators. In the high-PCE/methanol culture, methanol was primarily converted to acetate while releasing H_2 as a by-product [*10,27*]. The dechlorinators scavenged this H_2 pool as a direct source of electrons to reductively dechlorinate PCE and its daughter compounds. However, to sustain or transfer the culture with H_2 as the sole donor, filtered culture supernatant from the methanol-fed culture was required, presumably to supply nutritional factors [*16*].

Short-term studies by DiStefano [*14,15*] demonstrated that the direct addition of H_2 eventually resulted in failure in the absence of the addition of complex nutrient sources. On the other hand, later studies showed that the ability of the high PCE/methanol culture to support dechlorination without the addition of vitamin B_{12} was due to the presence of methanol-consuming acetogens (known to contain corrinoids) who thus supplied B_{12} to the dechlorinators [*15*]. Other co-contaminants in the culture apparently supplied yet other unidentified nutritional factors.

Apart from sustainability issues, the direct application of H_2 (an explosive gas that is only sparingly soluble in water) towards stimulating

dechlorination in the field proves to be both difficult and hazardous from an engineering point of view. Therefore, it is more practical to add complex nutrient sources that are converted to H_2 and / or support a more complex population to provide growth factors. Furthermore, field PCE concentrations are rarely inhibitory to methanogens, and henceforth, the challenge lies in the development of cultures that could sustain dechlorination at non-inhibitory PCE concentrations while being maintained on close-to-stoichiometric amounts of donor.

A schematic showing the interaction between fermenters, methanogens and dechlorinators in a mixed-dechlorinating culture operating under non-inhibitory and competitive conditions can be found in Figure 2.2.

In 1993, Stover compared the use of methanol to the use of non-methanogenic electron donors that are directly fermented to H_2 [*53*]. The PCE concentration administered was 110 μM (18 mg PCE/L, nominal concentration), a level somewhat more representative of those encountered at contaminated sites and within the problematic noninhibitory range (see Table 2.1). The non-methanogenic donors, ethanol and lactate supported dechlorination of PCE to VC and ETH for the 50-day study. Stover observed that when H_2 was high, both

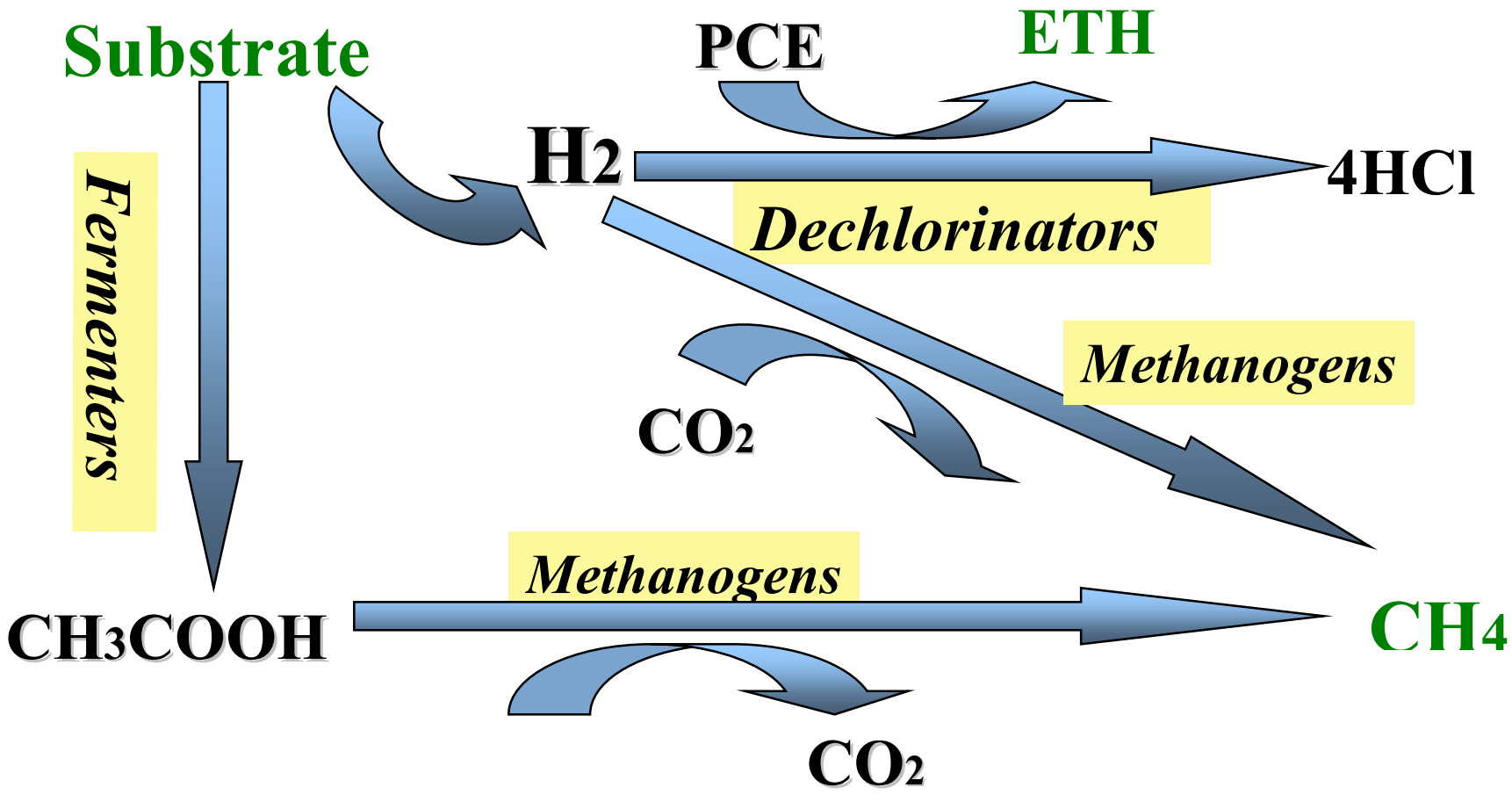

Figure 2.2 Interaction between Microbial Agents in Reductive Dechlorination under Non-Inhibitory PCE concentrations

dechlorination and methanogenesis occurred rapidly; but when H_2 levels were below about 4 x 10^{-4} atm, no methanogenesis occurred, while dechlorination continued. This suggested that dechlorinators could use H_2 at lower levels than could the methanogens, and thus, had a higher affinity and/ or lower threshold for H_2 use.

Smatlak et al. quantified the apparently different affinities for H_2 by methanogens and the dechlorinators through measurement of H_2 half-velocity coefficients, $K_{S(H2)dechlor}$ for the dechlorinators and $K_{S(H2)meth}$ for methanogens, in a mixed culture [*49,50*]. $K_{S(H2)dechlor}$ for H_2 use by dechlorinators averaged 100 nM while the $K_{S(H2)meth}$ for H_2-using methanogens averaged 960 nM. Ballapragada et al. reported $K_{S(H2)dechlor}$ for H_2 use by dechlorinators of 9 to 21 nM in a mixed culture containing methanogens and dechlorinators(s) [*3*].

Thermodynamic considerations suggest that a low $K_{S(H2)dechlor}$ could be universal for hydrogenotrophic dehalogenators. So much energy is available from the H_2-PCE couple (-168kJ/mol H_2), that organisms can gain energy for growth even when the H_2 concentration is very low – meaning that they could have a low threshold for H_2. It seems likely that such organisms would evolve kinetics of H_2 use that are favorable at these

low concentrations – i.e., that they would also have a relatively low $K_{S(H2)dechlor}$ value for H_2 use.

The order-of-magnitude difference in K_s values between dechlorinators and methanogens suggests that the dechlorinators could have a competitive advantage if the H_2 could be supplied in such a way as to be energetically and kinetically unfavorable for methanogenic use while being accessible to dechlorinators.

2. B. Stimulation of Reductive Dechlorination

Henceforth in order to simulate dechlorination in the field or the lab, the three scenarios under which hydrogenotrophic dechlorination occurs, based on PCE concentration and donor:PCE ratio would need to be understood. The characteristics of these three scenarios are summarized in Table 2.1. [*21*]

In the first scenario **(SCENARIO 1)**, PCE concentration is not inhibitory to competing organisms (<50 ppm), but available electron-donor level is many orders of magnitude higher than stoichiometrically required for dechlorination. In this case any donor added will – through

fermentation and the maintenance of a large, endogenously decaying biomass pool –produce enough trace, scavengeable H_2 to stimulate dechlorination of small quantities of chloroethenes. This can be true even of a donor such as methanol, which is directly used by methanogens. If PCE levels are relatively low, then even cometabolic reductive dechlorination may be a significant mechanism in transforming chloroethenes.

In the second scenario **(SCENARIO 2)**, PCE is present at high levels (>50 ppm) that are inhibitory to competing methanogens. In this case, relatively low ratios of donor may be applied, because most of the reducing equivalents will be available to the dechlorinators and the competition is minimized.

The third scenario **(SCENARIO 3)** is an "intermediate" situation where a significant (but non-inhibitory) amount of PCE is present (1-50 ppm), and donor is available at a similar level. Under these conditions, competition for limited donor by non-dechlorinators such as methanogens could dictate whether dechlorination is complete. Since dechlorinators have a significantly lower half-velocity coefficient (and probably threshold) for H_2 use than do methanogens, then application of H_2 donors that are fermented slowly, and under low H_2 partial pressures, selectively

contribute H_2 to dechlorinators, while minimizing that available for methanogens.

Table 2.1 Effects of PCE concentration and Donor:PCE Levels on Competition Scenarios between Dechlorinators and Methanogens

		Donor : PCE Ratio	
		Stoichiometric Amounts of Donor *(donor:PCE ratio ≤2:1 on H_2 equivalents basis)*	**Excess Amounts of Donor** *(donor:PCE ratio >>2:1 on H_2 equivalents basis)*
PCE Concentration	**Inhibitory (>50 ppm)**	Methanogenesis inhibited Dechlorination Favored **(SCENARIO 2)**	Methanogenesis inhibited Dechlorination Favored **(SCENARIO 2)**
	Non-inhibitory (<50 ppm)	Head-to-head competition between methanogenesis and dechlorination Preference towards dechlorination depends on partial pressure of hydrogen **(SCENARIO 3)**	Both methanogenesis and dechlorination allowed Dechlorination may occur as a result of cometabolism **(SCENARIO 1)**

In 1998, Fennell and Gossett compared alternative hydrogen donors for the reductive dechlorination of PCE [*18*]. Stimulation of reductive dechlorination was performed at the intermediate, "non-inhibitory" range **(SCENARIO 3)**. Four substrates were investigated: ethanol, lactate, butyrate and propionate. Based on bioenergetic calculations, fermentation reactions were most favorable (at H_2 partial pressures higher than 10^{-5} atm) for lactate, followed by ethanol, then butyrate and finally propionate.

Fennell and Gossett prepared separate, semi-continuously operated cultures that were enriched with one of each of the donors (ethanol, lactate, propionate and butyrate). The donor:PCE levels were under 2:1 on an H_2 equivalents basis and the PCE concentration was kept at 110 μM (within non-inhibitory range). Experimental results showed that slowly fermented substrates producing lower H_2 levels were more effective and persistent "selective" stimulators of dechlorination than rapidly fermented substrates producing higher H_2 levels.

Fennell and Gossett formulated a comprehensive model using Michaelis-Menten-type kinetics incorporating H_2 thresholds and thermodynamic limitations from Smatlak et al. on donor fermentations.

Mixed-culture behavior under a variety of conditions was fit well by the model [*19*].

2.C. Microbial Constituents in a Mixed-Dechlorinating Culture

In this section, the microbial constituents (dechlorinators, fermenters and methanogens) that are involved in the dechlorination process, or are in competition with it, are described. A schematic depicting the interactions among them is shown in Figure 2.2.

2.C.1. Dechlorinators

Many kinds of microorganisms are able to carry out reductive dechlorination to varying degrees of completion. They can be divided into two main types: 1) organisms that dechlorinate by cometabolism and 2) halorespiring organisms.

2.C.1.a. Definition of Cometabolism. Co-metabolism is defined as "the metabolism of a non-growth substrate in which no apparent benefit is accrued by the metabolizing organism" [*59*]. Co-

metabolism may occur through the action of enzymes with broad specificity as in the classic case of the methanotrophic enzyme, methane monooxygenase, which works on TCE, among other compounds [*33*]; or through reaction with the metal centers of various coenzymes or factors. Despite their low rates and inefficiency, these reactions may be important environmentally, since from site to site, it is not known whether halorespiring organisms will be present or whether fortuitous dehalogenation will be the dominant mechanism.

Many methanogens, such as *Methanosarcina* sp. strain DCM and *Methanosarcina mazei*, are able to perform reductive dechlorination of PCE to TCE [*17*] through cometabolic means. For example, when fed methanol, acetate, methylamine, or trimethylamine, *Methanosarcina* sp. strain DCM was able to dechlorinate PCE to TCE. The reaction was linked directly to CH_4 formation – when there was no CH_4 production, there was no dechlorination.

2.C.1.b. Definition of Halorespiration. Halorespiration is the usage of PCE or TCE as an electron acceptor along with an electron donor for energy generation. Halorespiring organisms exhibit much higher rates of dechlorination than do microorganisms or cell extracts carrying out fortuitous reactions [*28,29,54*]. As such, the focus on microbially-

mediated dechlorination is now directed toward isolation of halorespiring organisms.

Halorespiring organisms include *Desulfomonile tiedjei* [*53*], *Dehalospirillum multivorans* [*47*], *Dehalobacter restrictus* [*13*], Strain MS-1 [*48*], *Enterobacter* agglomerans [*48*], *Desulfitobacterium* sp. strain PCE1 [*25*], Strain TT4B [*31,32*] and *Dehalococcoides ethenogenes* [*38*].

2.C.1.c. *Dehaloccoides ethenogenes strain 195.* *Dehalococcoides ethenogenes* strain 195 was the dechlorinator used in the mixed culture for this study. It was isolated by Maymó-Gatell et al. in 1997 [*38*]. The organism was originally obtained from cultures that were developed from anaerobic digester sludge – i.e., the high PCE/methanol culture of Distefano et al. discussed earlier. It is a halorespiring dechlorinator.

Maymó-Gatell et al. performed MPN analysis on the high-PCE/methanol culture, and highly dilute cultures grown with PCE and H_2 were used as starting material to isolate the dechlorinator[*37*]. The dechlorinator uses acetate as a carbon source and it requires vitamin B_{12} at a rather high concentration – 0.05 mg/L. The exact identity of other required nutrients was not determined, and culture transfer continued to depend upon the addition of the complex nutrient sources yeast extract, anaerobic digester sludge supernatant, or extracts of cells from cultures

grown with more complex electron donors [*37*]. The dechlorinating organism was eventually isolated by Maymó-Gatell et al. in a microscopically pure form and was given the tentative name *Dehalococcoides ethenogenes* strain 195 [*38*]. The organism is a halorespiring organism and gains energy for growth from H_2 use, coupled with the reduction of PCE, TCE, cis-1-2-DCE, 1,1 DCE, or dichloroethane – but not from trans-1, 2, -DCE or VC (though it dechlorinates them) [*36*].

2.C.2. Fermenters

H_2 is an environmentally critical microbial product and substrate. Numerous organisms produce it through their fermentative activities or utilize it as an electron donor. H_2 production and subsequent competition for its use are important issues to consider in selecting an electron donor for dechlorination. Extensive literature sources have described organisms that produce H_2 through breakdown activities and the competition for the resulting H_2 by H_2 users, primarily between sulfate-reducing bacteria and methanogens.

Fermentations of alcohols and short-chain volatile fatty acids (VFAs) to H_2 are carried out by syntrophic, obligate proton-reducing

organisms. Substrates like butyrate and propionate are oxidized to acetate and CO_2 and the electrons liberated during the oxidation are then disposed of by the reductions of protons to H_2. Organisms that oxidize alcohols and VFAs with concomitant production of H_2 and acetate (see Figure 2.1) exist syntrophically with other organisms that utilize H_2 and acetate. They are dependent upon their syntrophic partners to remove these end products so that conditions are thermodynamically favorable for further metabolism. Obligate syntrophic proton reducers are fastidious anaerobes and have themselves proven difficult to culture and study. An excellent review of some of these processes has been provided by Schink [*46*].

Fermentation rates of different substrates depend highly on the H_2 partial pressure. A fermentation reaction is exergonic/favorable only when the ambient hydrogen pressure is lower than its "H_2 ceiling". For example, lactic acid, ethanol, butyric acid and propionic acid are exergonic under H_2 partial pressures (ceilings) of less than about 1, 0.1, $10^{-3.5}$ or $10^{-4.4}$ atm, respectively. It is generally true that substrates with higher H_2 ceilings will ferment faster than those with lower H_2 ceilings. Yet external factors may apply; for example, ethanol ferments faster than lactic acid [*18*].

2.C.3. Methanogens

Methanogens are ubiquitous in fermentative environments. Divided into two groups, they either generate methane through hydrogenotrophic or acetotrophic means. Some have dechlorinating capabilities through cometabolism [*17*]. Despite this, methanogens appear to have significantly less tolerance than halorespiring dechlorinators towards PCE and its daughter compounds [*15,16*]. Common conditions in the field would pit methanogens against dechlorinators in a head-to-head competition for hydrogen. However, because of the order-of-magnitude difference in their half-velocities constants for hydrogen utilization [*50*], low H_2 partial pressures sustained by slowly fermented substrates are more likely to favor dechlorination over methanogenesis.

2.D. Application of Different Electron Donors in Stimulating Reductive Dechlorination

2.D.1. Use of Electron Donors with High-Solubility in Reductive Dechlorination

As mentioned earlier, methanol was used in early research in reductive dechlorination as an electron donor. Although it is able to dechlorinate PCE to ETH and VC, it allows the direct production of methane without fermentation. As a result the amount of electrons equivalents channeled towards fermentation and subsequently dechlorination is substantially reduced and its donor efficiency is rather low – unless PCE, TCE levels are sufficiently high to inhibit methanogens. At intermediate, noninhibitory PCE concentrations, when methanol was fed, a "spiral to failure" was observed where methanol simply supported the formation of more and more methanol- and H_2-using methanogens, which eventually took over the culture and left few reducing equivalents for dechlorinators to scavenge [*52*]. Dechlorination eventually failed in these systems.

Other non-methanogenic donors such as ethanol, lactic acid, propionic acid and butyric acid were tested by Fennell and Gossett [*18*] under intermediate, non-inhibitory conditions. Over the long-term, all substrates stimulated nearly equally the dechlorination of PCE to VC and ETH; however, stimulation of methanogenesis differed among the donors, with the highest amount of methane generated in cultures fed with ethanol, followed by lactate, then butyrate and finally propionate.

During short-term tests, patterns of donor fermentation and H_2 production and consumption were significantly different between the donors. Fennell found that when the cultures were fed with amounts stoichiometrically sufficient to completely dechlorinate PCE, half the H_2 released during ethanol fermentation was used methanogenically with the remainder channeled to incomplete dechlorination; however, only one percent of the H_2 released during propionate fermentation was used methanogenically and the remainder was used for complete dechlorination. The lack of observed differences in dechlorination with different H_2 donors during long-term studies was caused by routine addition of a nutritional supplement (fermented yeast extract) that also contained butyrate and propionate. Fermented yeast extract was not added in the short-term studies.

A comparison between the various donors under short-term studies is summarized in Table 2.2.

Table 2.2. Comparison of Ethanol, Lactic Acid, Propionic Acid and Butyric Acid under Time-Intensive Studies (donor:PCE ratio = 2:1 H_2 basis) [*18*]

	H_2 production	**Dechlorination**		**Methanogenic Competition (CH_4 produced)**
		Dechlorination Rate	**Completeness of Degradation**	
Ethanol	Small initial burst at 3000-5000 nmol/bottle	Fast rate of dechlorination during initial burst. Rate reduced drastically afterwards	Degradation to Vinyl Chloride	Produced rapidly during initial burst but ceased as H2 partial pressure dropped
Lactic Acid	Small initial peak at 250 nmol/bottle for 1:1 donor:PCE ratio Initial peak at 3000 nmol/bottle for 2:1 ratio	Fast rate of dechlorination during initial burst. Slower rate afterwards perpetuated by pool of propionic acid	Degradation to Vinyl Chloride	Persisted at low rate throughout experiment
Butyric Acid	Steady H2 production at low 20-30 nmol/bottle levels	Slow but slightly faster than Propionic Acid.	Mostly degraded to Vinyl Chloride Some TCE left	Small amounts of methane produced

Propionic Acid	Slow steady H_2 production at around 20 nmol/bottle l levels	Slow but steady throughout the whole process	Degradation to Vinyl Chloride	Trace methane produced

2.D.2. Use of Electron Donors with Low-Solubility in Reductive Dechlorination

Application of soluble substrates in the field towards enhanced in-situ bioremediation entails injection and recovery pumping to facilitate transport/distribution of donor (see Figure 2.3). In terms of cost, equipment and operation, this mode of stimulating reductive dechlorination has difficulty in competing with conventional pump and treat methods; although reductive dechlorination stimulates in-situ bioremediation and promises more complete and thorough clean-up.

As such, many remedial projects for chloroethenes have now shifted towards applying low-solubility or even solid substrates. The advantage of solid substrates is that, due to their relatively immobile state, they can be introduced in the treatment zone in large amounts – thus avoiding continuous injection and recovery. Their disadvantage, of course, is the difficulty of distributing reducing equivalents throughout the treatment zone. Thus solid or low-

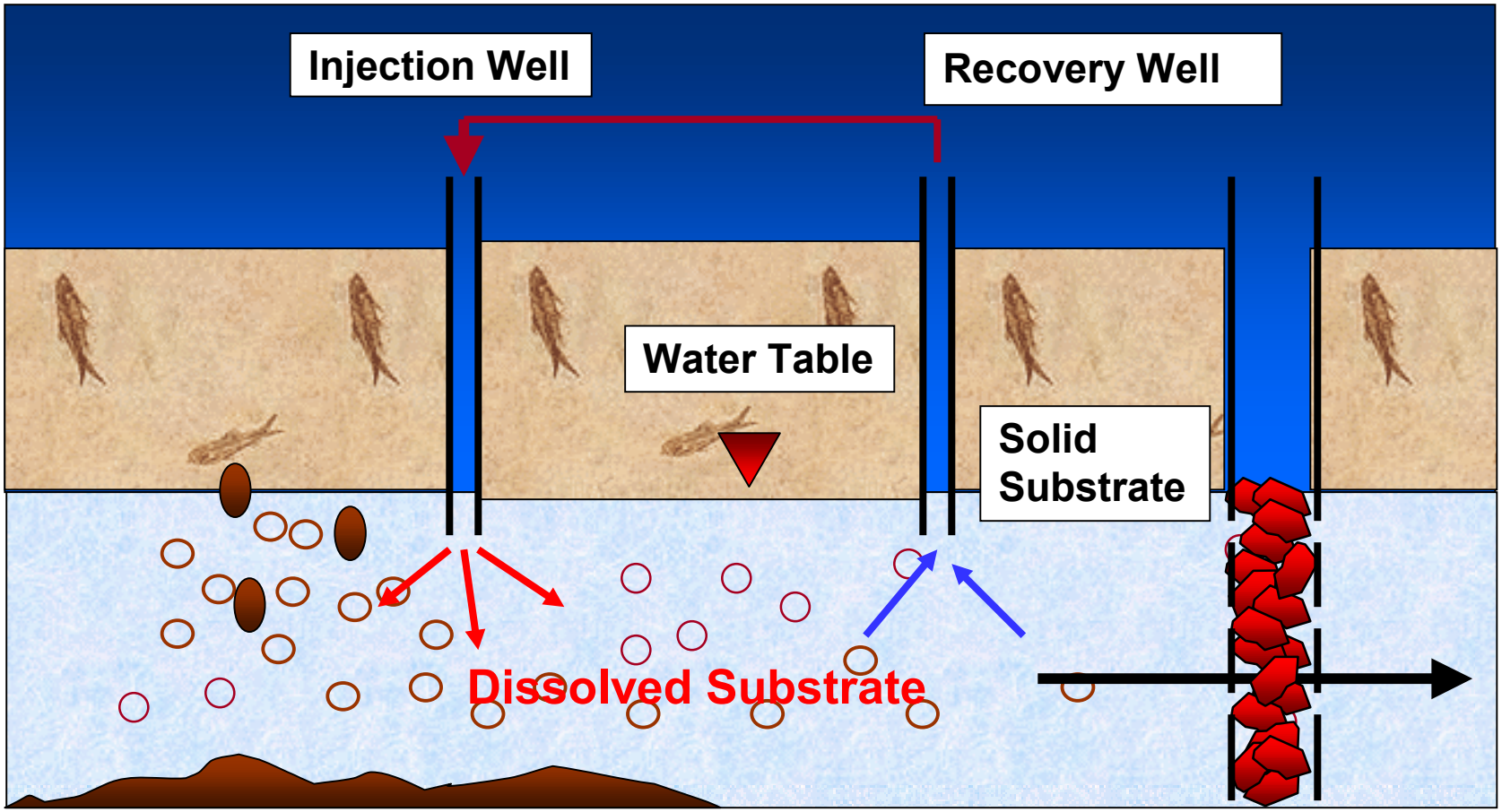

Figure 2.3 Conventional Methods of Stimulating Reductive Dechlorination in the Field

solubilitysubstrates might be most beneficially used in "bio-barrier" plume-containment applications. For example, trenches could be excavated and backfilled with a mixture of such substrates and soil/sand particles. If carefully engineered, donor availability (i.e. donor:PCE ratio) would be regulated at stoichiometric amounts by the slow-dissolving mechanism of the substrate. Limited donor availability would mean that competitive advantage would be given to dechlorinators because of slow production of H_2. Meanwhile, biofouling caused by over-availability of dissolved substrate would also be kept to minimum.

Currently many solid substrates have been investigated as possible candidates as low-solubility substrates. Among these are chitin (a complex natural polymer found in insect exoskeletons), Hydrogen Release Compound®, HRC® (an industrial compound specially designed to cause slow production of hydrogen), and vegetable oil (henceforth abbreviated as vegoil, a food-grade, non-toxic, low solubility solid/liquid).

Chitin is a linear polysaccharide consisting of (1-4)-linked 2-acetamido-2-deoxy-β -D-glucopyranose [*30*]. In nature, chitin is similar to cellulose both in chemical structure and in biological function as a structural polymer. The crystalline structure of chitin has been shown to be similar to cellulose in the arrangements of inter- and intrachain

hydrogen bonding. Its complex and interlinked structure yields stability and makes chitin even somewhat recalcitrant to natural degradation. This makes it relatively less accessible to fermenters. However when introduced in large amounts, the gross amount of hydrogen produced might be able to stimulate reductive dechlorination.

HRC® is a lactic acid polymer bonded to glycerol [*44*]. The lactic-acid polymer is formed by ester-linkages between adjacent lactic acid monomers (each lactic acid molecule contains one alcohol and acid group). Fermentation first requires hydrolysis of such bonds to release lactic acid monomers. Slow fermentation of lactic acid is expected to yield low hydrogen partial pressure, favorable to dechlorinators.

2. E. Vegoil as Electron Donor

The electron donor used in this study is vegetable oil. Physical state of vegoil varies – most vegetable oils are liquid under room condition while numerous kinds (such as Palm Kernel Oil) have melting points higher than 70^{o}F (21^{o}C). In the experimental setup of this experiment, vegoil exists as a liquid and hence behaves like a LNAPL sitting on top of the culture solution.

Vegoil is mainly composed of triacylglycerols which typically consist of long-chain fatty acids of lengths 8 to 18 carbons. It is presumed that once triacylglycerols have been de-esterified, the long-chain fatty acids would be freed into the surrounding aqueous phase. Typical fermenters would be able to utilize them by applying the same mechanism of beta-oxidation as they would with VFAs longer than 2 carbons.

Other advantages of vegoil may lie in it being a complex and comparatively less well-defined substrate. Other than the triacylglycerols, unrefined vegoils may contain compounds that could be nutrients. It is hoped that such nutrients may include some of those found in yeast extract and vitamin B_{12}. Even if not directly present in vegoil, nutrients may be generated by the complex microbial community expected to arise on a complex vegoil substrate. In previous research, stimulation of dechlorination using well-defined, neat substrate (such as methanol, ethanol, lactate and butyrate, etc.) eventually deteriorated over the long term if the addition of copious amounts of yeast extract and vitamin B_{12} were omitted [*18*]. On the other hand, unrefined vegoils may contain inhibitory compounds that may affect the dechlorinating performance of

the culture. Both these aspects of additional compounds in vegoil will be examined in this study.

2.E.1. Issues of Applying Vegoil in the Field

When applied to the field, vegoil is expected to form an LNAPL, some of which may end up on the groundwater surface. This LNAPL could possibly partition the surrounding dissolved chloroethenes into the non-polar vegoil phase and possibly lower concentrations downstream (see Figure 2.4). Therefore it would be interesting to know how dechlorination of the sequestered chloroethenes would proceed. It is known that dechlorination occurs in the aqueous phase and is dependent on the dissolution for PCE and its daughter compounds into groundwater.

Carr, et al. did a study on the effect of dechlorinating bacteria on the longevity and composition of PCE-containing NAPLs under equilibrium dissolution conditions [*11*]. Experiments were conducted in continuous-flow stirred-tank reactors (CFSTRs) containing a mixed PCE dechlorinating culture and a model NAPL consisting of PCE and tridecane, a non-reactive NAPL. Comparisons between biotic and abiotic CFSTRs demonstrated that dechlorination resulted in a factor of 14 increase in PCE removal rates from the NAPL. The formation of

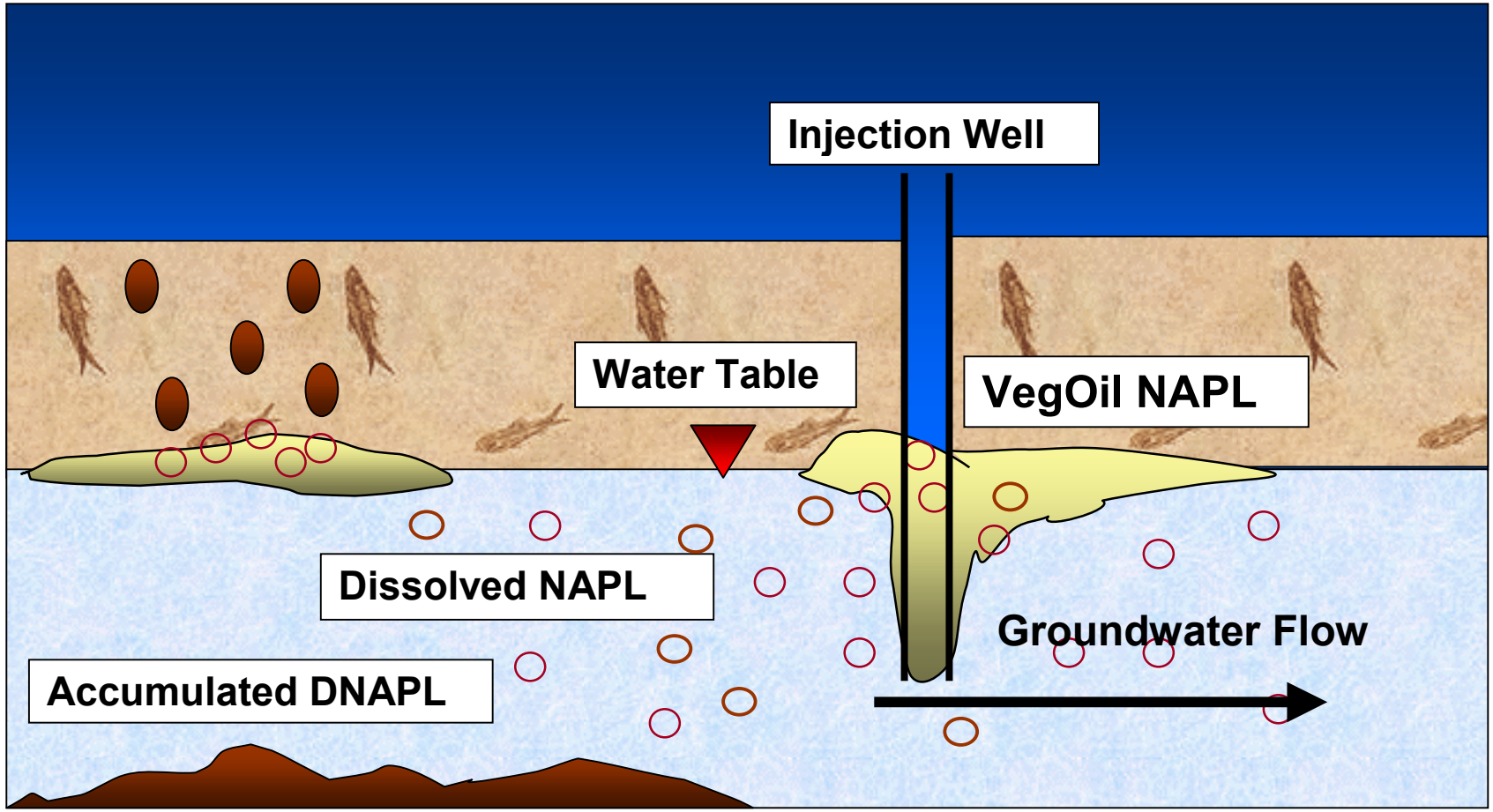

Figure 2.4 Possible Methods of Stimulating Reductive Dechlorination in the Field with Vegoil

dechlorination daughter products trichloroethene and *cis*-dichloroethene were observed, and *cis*-dichloroethene was not dechlorinated further.

Partitioning of daughter products between phases caused temporal changes in the chlorinated ethenes distribution within the NAPL. The combined effects of dissolution and dechlorination on the removal of chlorinated ethenes from the NAPL were described using a mathematical model that approximated dechlorination as a pseudo-first-order process. Partition coefficients decreased as the chlorinated ethene became more reduced due mainly to their increasing aqueous solubilities. Thus, dechlorination in the presence of a PCE-containing NAPL will yield reduced species that partition more strongly into the aqueous phase, and an increase in the total chlorinated ethenes removal rate from the NAPL is possible.

The purpose of Carr et al. in concocting a NAPL consisting of PCE and a conservative hydrocarbon was to imitate a mixed organic waste. It is observed that pure phase NAPLs are not commonly encountered and that many chlorinated solvent spills occur in the presence of fuels or other organics such as mineral spirits [*40,53,57*]. Yet BTEX and other constituents that are commonly found in mixed NAPLs and that may serve as electron donors were omitted from the NAPL as their dissolution

would have significantly increased the complexity in describing observed results and transient NAPL composition. Although experiments with NAPLs containing an internally supplied electron donor, such as a vegoil layer containing PCE, were not attempted by Carr, et al., they are of great field relevance and warrant investigation.

It is projected that in a vegoil culture where the NAPL itself is being reacted, the mass transfer rate of PCEs and its daughter compounds from the NAPL phase to the aqueous phase would be greatly enhanced. Breaking down of the vegoil layer would release sequestered chloroethenes into the surrounding aqueous phase. Meanwhile breakdown products of vegoil such as hydrogen and volatile fatty acids could dechlorinate PCE further and increase the drive for the dissolution of more chloroethenes.

A study on such interactions would be highly relevant to assessing the overall dechlorination ability of vegoil and its effect on the fate and transport of chloroethenes. Although the main emphasis of this study is only to investigate vegoil's ability as a fermentable substrate based on microcosm studies, vegoil's properties as a NAPL are expected to have a large effect on the experimental results. Such potential effects would be

very useful in helping one understand the behavior of vegoil in the field. It is projected that once stimulation of reductive dechlorination has been successfully demonstrated, modeling of vegoil dechlorination under field conditions would be followed-up subsequently.

CHAPTER THREE

MATERIALS AND METHODS

3.A. Experimental Overview

Microcosms were prepared using an anaerobic mixed culture as the source culture. The culture consisted of an established dechlorinator — *Dehalococcoides ethenogenes* strain 195, various fatty-acid fermenters and various methanogens. This culture had been continuously maintained on PCE and butyrate for several years by J. M. Gossett. Four types of substrates were investigated — 3 vegetable oils and 1 commercial hydrogen donor. The types of vegetable oil ("vegoils") were Palm Kernel Oil, Crude Soybean Oil and Refined, Bleached and Deodorized Soybean Oil (Commercial Soybean Oil). The commercial donor was Hydrogen Release Compound (HRC®) from Regenesis, Inc., a polylactate tri-ester.

Aliquots of the culture were anaerobically transferred to 160-mL serum bottles. Bottles were amended with the following treatments:

a) PCE only

b) PCE + substrate

c) PCE + substrate + yeast extract + vitamin mixture

d) PCE + yeast extract + vitamin mixture

Bottle-types (a) and (d) served as unfed controls to allow the role of the substrates (vegoil or HRC) to be assessed.

Each serum bottle was spiked with 11 μmol of PCE at the beginning of the experiments. For the vegoil bottles: once the PCE level had dropped to zero, the bottle was "re-spiked" with another 11 μmols of PCE. This re-spiking process was repeated as required throughout the 140-day monitoring period. HRC® bottles were only spiked once at the beginning. 50 mg of vegoil or 100 mg HRC® was fed to the bottles at the beginning of the experiments. Since most equivalents were channeled towards methanogenesis, methane levels were used to indicate the status of consumption of substrate. More specifically, when methane levels ceased to rise (or "plateaued"), substrate was assumed to be completely consumed. Consequently, the samples were "re-fed" with another 50 mg-dose of substrate (HRC® samples were not re-fed). Directly before the re-feeding process, a "purging" process was executed – where an anoxic gas was blown into the culture to remove remaining chloroethenes and methane. After that, 10 mL of the culture was replaced by 10 mL of fresh Basal Media before 50 mg of vegoil was added. Throughout the 140-day

monitoring period, the "purge and re-feeding" process was only carried out once per sample (excluding unfed controls and HRC® samples).

Time-course profiles of hydrogen gas, chloroethenes, methane were monitored every four days. Volatile Fatty Acids (VFAs) were measured on days 8, 40, 128. Biomasses were measured at the end of the monitoring period using the Total Kjeldahl Nitrogen method.

3.B. Source Culture

A low-PCE/butyric acid source culture had been continuously maintained by J.M. Gossett for 5 years. This culture served as inoculum for all the experiments in this thesis. Details of the protocol for operating and maintaining the source culture are described by D. Fennell [*20*]

The source culture was maintained at 35°C and was regularly monitored for chloroethenes, CH_4 and pH to ascertain culture health. The culture was brought to a PCE concentration of approximately 110 μM or 18 mg/L (nominal concentration, ignoring partitioning to the headspace) every 3 to 7 days. Butyric acid was added at a 10:1 ratio to PCE on an equivalents basis. For purposes of this research, equivalence was defined on a $[CO_2]$ basis. If one assumes complete oxidation to yield CO_2

molecules, 20 equivalents exist per mole of butyrate. The amount added at each feeding was 440 μM (38.72 mg/L). An anoxic yeast extract (YE) solution containing 50 g yeast extract/L was added at each feeding to obtain 20 mg YE/L in the culture. Vitamin solution (described below) was added at the rate of 0.5 μL/mL culture.

3.B.1. Basal salts medium.

The source culture was grown in a basal salts medium which had been used to develop and work with a mixed culture of butyrate fermenters, methanogens and dechlorinators. The solution was adapted by Freedman [*22*] from one described by Zeikus [*62*] for methanogens. The composition of the medium is shown in Table 3.1. The solution was prepared in 15-L batches and was stored at 35^{o}C under a pressurized anoxic atmosphere to prevent the infiltration of air.

Table 3.1 Basal salts medium

Compound	Quantity (per L distilled water)
NH_4Cl	0.2 g
$K_2HPO_3 \cdot 3H_2O$	0.1g
KH_2PO_4	0.055 g

$MgCl_2 \cdot 6H_2O$	0.2 g
Resazurin	0.001 g
Trace Metal Solution†	10 mL
$FeCl_2 \cdot 4H_2O$	0.1g
$Na_2S \cdot 9H_2O$	0.5 g
$NaHCO_3$	6.0 g

The first seven items were added, and an N_2 purge was maintained until the solution changed from blue to pink. The composition of the purge gas was changed to 80%N_2/20% CO_2 purge and then the remaining two items were added quickly. The solution was black and changed to pink if oxygen was present.

† Trace Metal Solution: 0.1 g/L $MnCl_2 \cdot 4H_2O$; 0.17 g/L $CoCl_2 \cdot 6H_2O$; 0.10 g/L $ZnCl_2$; 0.251 g/L $CaCl_2 \cdot 2H_2O$; 0.019 g/L H_3BO_3; 0.05 g/L $NiCl_2 \cdot 6H_2O$; 0.02 g/L $Na_2MoO_4 \cdot 2H_2O$. Adjusted to pH 7 with 8 N NaOH.

3.B.2. Yeast extract solution.

Yeast extract served as a trace nutrient source for the low-PCE/butyrate culture. To 100 mL of distilled water, 5 g of yeast extract

powder (Difco Laboratories) was added. The solution was purged for 30 min with anoxic gas (80% N_2/ 20% CO_2 – scrubbed with titanium citrate solution – see section 3.C.2.) and was then capped with a gray-butyl septum and aluminum crimp cap. The solution was stored refrigerated. Prior to removing yeast extract solution from the bottle, the same volume of anoxic gas was delivered to prevent a vacuum from forming in the bottle.

3.B.3. Vitamin solution.

An anoxic, aqueous vitamin solution described by D. Fennell [*20*] was added to some bottles where noted. The solution was prepared with crystalline or powdered forms of the vitamins (all 99.9%, Sigma Chemical Co.), and purged with anoxic gas as in the preparation of YE described earlier. The solution was refrigerated inverted for storage. The composition of the vitamin solution is presented in Table 3.2.

Table 3.2. Vitamin solution for amendment of cultures.

Constituent	Quantity (mg/L)
d-biotin	20
folic acid	20
pyridoxine hydrochloride	100
thiamin hydrochloride	50
riboflavin	50
nicotinic acid	50
DL-calcium pantothenate	50
Vitamin B_{12} (cyanocobalamin)	10
p-aminobenzoic acid	50
lipoic acid	50

3.C. Serum Bottle Studies

In this section, general information about serum bottle set-up and handling is presented. All serum bottle tests were performed at 35^{o}C under orbital agitation.

3.C.1. Set-up of Serum Bottles from Source Cultures

Experiments were performed in 160-mL serum bottles containing 100 mL of culture and 60 mL of headspace. The bottles were sealed with Teflon®- backed, gray-butyl rubber septa (Wheaton Industries) and aluminum crimp caps.

To prepare microcosms directly from source culture, a 4-L jar was used to transport the source culture from the source culture reactor to an anaerobic glovebox in the following manner: First, the 4-L jar was filled with distilled water and inverted into a large container of distilled water. Second, a cannula delivering anoxic gas was introduced into the bottle to displace the distilled water. Then the jar was removed from the container and placed upright while still purging. A source culture reactor that had been purged and pressurized was used to deliver the required amount of culture into the 4-L jar; while still under anoxic purge.

Once the required amount was delivered, the 4-L jar was sealed with a rubber stopper. The rubber stopper was secured in place with retaining clamps and bolts. The jar was transported into an anaerobic glovebox containing only nitrogen gas and trace amounts of hydrogen gas (typically less than 1% H_2). All culture transfers to individual serum

bottles were performed inside the glove box. A graduated cylinder was used to transfer 100 mL of the source culture to each individual serum bottle.

The serum bottles, each containing aliquots of the culture (100 mL), were amended with the following treatments shown in Table 3.3. Note that yeast extract was added in doses of 40.4 μL/bottle (20 mg/bottle) while vitamin solution was added in doses of 50.9 μL/bottle. Palm Kernel Oil, Crude Soybean Oil and Refined, Bleached and Deodorized Soybean Oil were added in 50 mg/bottle doses. The serum bottles were initially spiked with 11 μmol of PCE per bottle and were re-spiked with the same amount whenever PCE concentrations in the bottles dropped to zero.

Table 3.3. Setup of Serum Bottle Studies

Sample Type	Samples
Unfed controls	• PCE only • PCE + yeast extract + vitamin mixture
Vegoil Samples a) PCE + vegoil	• PCE + Palm Kernel Oil • PCE + Crude Soybean Oil • PCE + Refined, Bleached Deodorized

	Soybean Oil
b) PCE + vegoil + yeast extract +vitamin mixture	• PCE + Palm Kernel Oil + Yeast Extract + Vitamin Mixture • PCE + Crude Soybean Oil+ Yeast Extract + Vitamin Mixture • PCE + Refined, Bleached Deodorized Soybean Oil+ Yeast Extract + Vitamin Mixture
c) HRC® Samples	• PCE + HRC® • PCE + HRC® + Yeast Extract + Vitamin Mixture

50 mg of the vegoil specific for each bottle was added by volumetric means. Prior to addition, the density of each vegoil was measured by weighing 100 mL samples of each type of vegoil. The volumes added are shown in Table 3.4. Digital pipettes (accurate to 1 μL) with disposable tips were used to add the required amount to the serum bottles. The bottles were then capped with gray-butyl, Teflon®-lined septa (Wheaton Industries) and crimped with aluminum caps. Triplicates were prepared for each of the bottle types stated above.

Table 3.4. Volumes Corresponding to 50 mg

Type of Vegoil	Volume Corresponding to 50 mg
Palm Kernel Oil	58.2
Crude Soybean Oil	56.4
Refined, Bleached and Deodorized Soybean Oil	55.1

For comparison purposes, one set of serum bottles was prepared with 100 mg of HRC® as the electron donor. Monitoring of the HRC® set was discontinued once it had been established that dechlorination was supported quite well on this substrate. This also served to demonstrate the health of the culture.

3.C.2. Protocol for Serum Bottle Operation

Neat PCE was added in spikes of 11 μmol by microliter syringe (Hamilton Company). When required by the bottle type, yeast extract was added as a nutrient source in an anoxic aqueous form. An anoxic aqueous mixture of vitamins was added using a gas-tight, locking syringe (Dynatech Precision Sampling Corp.).

The time-course profiles of volatile fatty acids (VFAs — acetic, propionic, butyric, isobutyric, valeric, isovaleric, and hexanoic), H_2, chloroethenes, and methane were monitored after setup. Every fourth day during long-term operation, a 0.1-mL headspace sample was removed from each bottle for analysis of dechlorination products and methane. 0.5 mL of the serum culture was removed every 40 days (on average) and measured for volatile fatty acids (VFAs).

Whenever the PCE level in a bottle was reduced to zero by dechlorination, an extra 11 μmol would be introduced. Methane production was used to reflect the course of substrate consumption because most electron equivalents from the vegoils were channeled into methanogenesis.

Once methane in a bottle had reached asymptotic level it was deduced that substrate in the bottle had been depleted. The depleted bottles then went through a "purging and re-feeding" process. An anoxic gas mixture (80% CO_2, 20 % N_2) that has been scrubbed with titanium citrate solution to remove oxygen was used to purge the bottles. The solution was prepared by adding 10 mL of 20% titanous chloride solution (Fisher Scientific Co.), 12.5 g sodium bicarbonate (Fisher Scientific Co.), and 4.412 g of citric acid, trisodium salt dihydrate (99%, Aldrich

Chemical Co. Inc.) to 1 L of distilled water. The titanium (III) citrate complex forms a violet/blue solution that loses its color upon oxidation [*61*].

Each bottle was purged with the anoxic gas mixture for 10 mins to remove chloroethenes and methane. After purging and re-feeding, 10 mL of the culture from each bottle was removed and discarded using a 25-mL glass syringe (Wheaton Company). Then 10 mL of fresh basal medium was added anaerobically via another 25-mL glass syringe.

50 mg of the respective vegoil was added volumetrically by using a digital pipette (accurate to 1 μL). (Note: HRC®-fed bottles were not re-fed). The bottles were then capped and sealed with Teflon®- backed, gray-butyl rubber septa (Wheaton Industries) and crimped with aluminum crimp caps.

After the exchange, the bottles were fed neat PCE, YE and vitamin solution (if required by bottle type). During long-term operation, bottles were incubated in a 35°C walk-in chamber, in a slanted, inverted position on an orbital platform shaker (Innova 2000, New Brunswick Scientific Co., Inc.) at 165 rpm.

Over the monitoring period of 140 days, purging, re-feeding and wasting of the vegoil cultures was only performed once per each vegoil

substrate (purging, re-feeding and wasting was not performed for HRC® cultures).

If we assume that the empirical formula of vegoil was $C_8H_{16}O$ (regardless of which kind), each "mole" of vegoil should yield 46 electron equivalents upon complete oxidation to carbon dioxide. Hence 50 mg of vegoil should yield a total of 17,970 μeqs. This corresponds to a maximum methane level of 2.25 mmol (ignoring equivalents channeled to biosynthesis). The unfed controls: PCE only and PCE + yeast extract + vitamins served as controls to allow the role of the oils to be assessed.

3.D. Analytical Methods

3.D.1. Reagents and Solutions

Palm Kernel oil (Edible Oil Technology), Crude Soybean Oil (Edible Oil Technology), Refined, Bleached and Deodorized Soybean Oil (Edible Oil Technology), Hydrogen Release Compound® or HRC® (Regenesis™), and PCE (Eastman Kodak Co., 99%) were used as direct culture amendments and for preparation of analytical standards. The vegoils normally exist as pure triacylglycerols. The typical fatty acid

composition of the various vegoils is shown in Table 3.5. Note that crude and refined, bleached and deodorized soybean oils contain the same fatty acids; hence there is only one entry for both oils.

Table 3.5 Compositions of Vegetable Oils (from Edible Oil Technology)

Fatty Acid		Soybean	Palm Kernel
Caprylic	(C8:0)	--	5.1
Capric	(C10:0)	--	3.9
Lauric	(C12:0)	--	39.9
Myristic	(C14:0)	0.1	12.5
Palmitic	(C16:0)	10.6	8.9
Palmitoleic	(C16:1)	trace	--
Stearic	(C18:0)	4.0	2.5
Oleic	(C18:1)	23.2	23.4
Linoleic	(C18:2)	53.7	3.7
Linolenic	(C18:3)	7.6	--
Arachidic	(C20:0)	0.3	0.1

HRC® is commonly known as glycerol tripolylactate. Its IUPAC name is propanoic acid, 2-[2-[2-(2-hydroxy-1-oxopropoxy)-1-oxopropoxyl-1-oxopropoxy]-1,2,3-propanetriyl ester.[*44*] Commercial literature [*43*] proclaims it as an effective electron donor in stimulating dechlorination. Thus the monitoring of HRC® cultures served dual purposes: 1) to ascertain the health of the culture and 2) to act as comparison against the vegoils.

Glacial acetic acid (Mallinckrodt, Inc., 99.5 to 100.5%), isobutyric acid (Fisher Scientific Co., 99%), isovaleric acid (Aldrich Chemical Co., 99%), and hexanoic acid (Aldrich Chemical Co., 99.5%) were used for preparation of analytical standards. TCE (Fisher Scientific Co., 99%), cis-1,2 DCE — (obtained in neat form in ampules from Supelco, Inc.), VC (Matheson Gas Products, 99%), ETH (Matheson Gas Products) and CH_4 (Scott Specialty Gases) were used for preparation of analytical standards.

3.D.2. Chlorinated Ethenes, CH_4 and H_2 Analyses

Analysis of PCE, TCE, DCEs, VC, ETH, methane and H_2 was performed with two Perkin-Elmer Corporation model 8500 gas

chromatographs which were equipped with flame ionization detectors (FID) and a stand-alone Trace Analytical Corp. reduction gas detector (RGD). A single 0.1-mL headspace sample removed from the serum bottle headspace via a gas-tight, locking syringe (Dynatech Precision Sampling Corp.) and was injected into the system. The outputs from the FIDs were integrated by their respective GC integration systems and the results from each were output to the screen and recorded. The RGD detected the hydrogen level in the sample and output it to a Perkin-Elmer LCI-100 integrator.

When a sample was injected, the GC system was activated and followed a programmed method developed by D. Fennell [*20*] to actuate the switching valves at specific times, controlling to which detector the separated compounds were directed. Except for a few minor changes, the details of the method used in this analysis were adapted exactly from the protocol described by Fennell.

3.D.3. Calibration for Chlorinated Ethenes, CH_4 and H_2 Analyses

Calibration factors for PCE, TCE, cis-DCE, VC, ETH and CH_4, were determined at the beginning of the microcosm studies and also once

every one or two months to ensure accuracy. Four 160-mL serum bottles that contained 100 mL of distilled water were used for calibrating PCE, TCE, cis-DCE, VC and methane. Concentrations of the mentioned chemicals were added via a gas-tight, locking syringe (Dynatech Precision Sampling Corp.) to a series of four standard bottles in the ratio of 1:2:3:4. A summary of the amount of chemicals added per standard bottle is presented in the Table 3.6.

Table 3.6 Summary of Chemicals Added to Calibration Standards

	Standard 1	**Standard 2**	**Standard 3**	**Standard 4**
PCE $_{(liq)}$	0.24 µL (2.34 µmol)	0.48 µL (4.88 µmol)	0.76 µL (7.42 µmol)	1.00 µL (9.76 µmol)
TCE $_{(liq)}$	0.24 µL (2.67 µmol)	0.48 µL (5.57 µmol)	0.76 µL (8.46 µmol)	1.00 µL (11.14 µmol)
DCE $_{(liq)}$	0.24 µL (3.08 µmol)	0.48 µL (6.42 µmol)	0.76 µL (9.76 µmol)	1.00 µL (12.85 µmol)
VC $_{(gas)}$	250 µL (20.5 µmol)	500 µL (40.9 µmol)	750 µL (61.4 µmol)	1000 µL (81.8 µmol)
ETH $_{(gas)}$	250 µL (20.5 µmol)	500 µL (40.9 µmol)	750 µL (61.4 µmol)	1000 µL (81.8 µmol)
CH_4 $_{(gas)}$	500 µL (40.9 µmol)	1000 µL (81.8 µmol)	1500 µL (122.8 µmol)	2000 µL (163.6 µmol)

The temperature and barometric pressure were noted at the time of the transfer of VC, ETH, or CH_4 and the moles of each gas added were determined from the ideal gas law.

The standard bottles were allowed to equilibrate at 35^{o}C in an orbital shaking water bath (Gyrotary Water Bath Shaker Model G76D, New Brunswick scientific Co, Inc.) at 165 rpm for at least a day and then were analyzed with a 0.1 mL injection volume to the GC system. The calibration factor for each component (μmol component per peak area of output) was calculated by means of regression through the data from the four standard bottles. The calibration factors for 0.1-mL injections had r^2 values ranging from 0.95 to 0.99.

An investigation of the partitioning of chloroethenes into the vegoil layer was made by calibrating with standard bottles that contained both water and vegoil. 100 mL of water and 100 mg of each type of vegoil or HRC® were used in preparing these standard bottles and the bottles were prepared in the same way as described earlier in this chapter. The resulting calibration curves can be observed in Figures 3.1 and 3.2. (Note that PK Olein stands for palm kernel oil while Soybean RBD stands for refined, bleached and deodorized soybean oil).

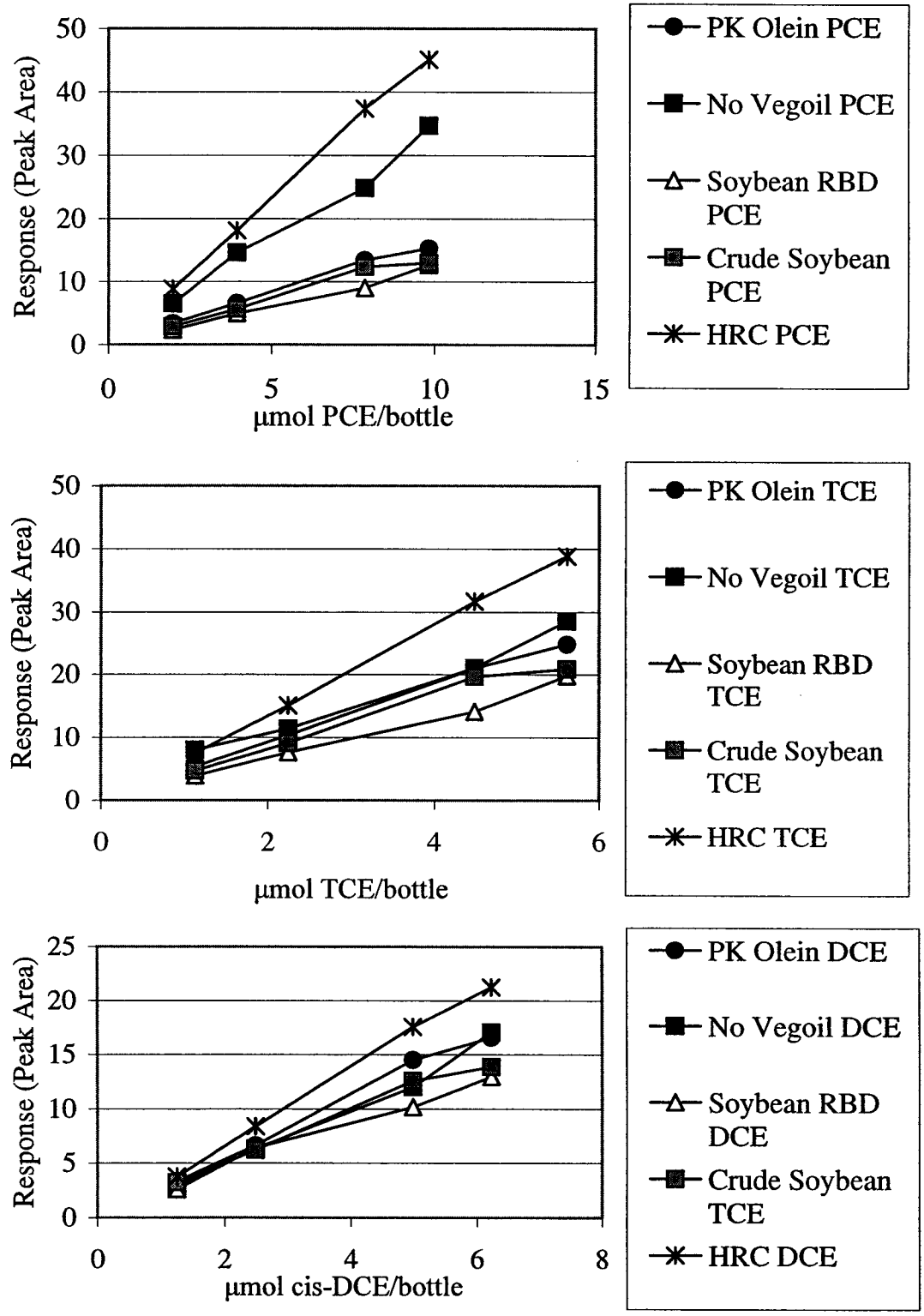

Figure 3.1 Comparison of PCE, TCE and cis-DCE Calibration Curves for Different Vegoil-Amended Standard Bottles

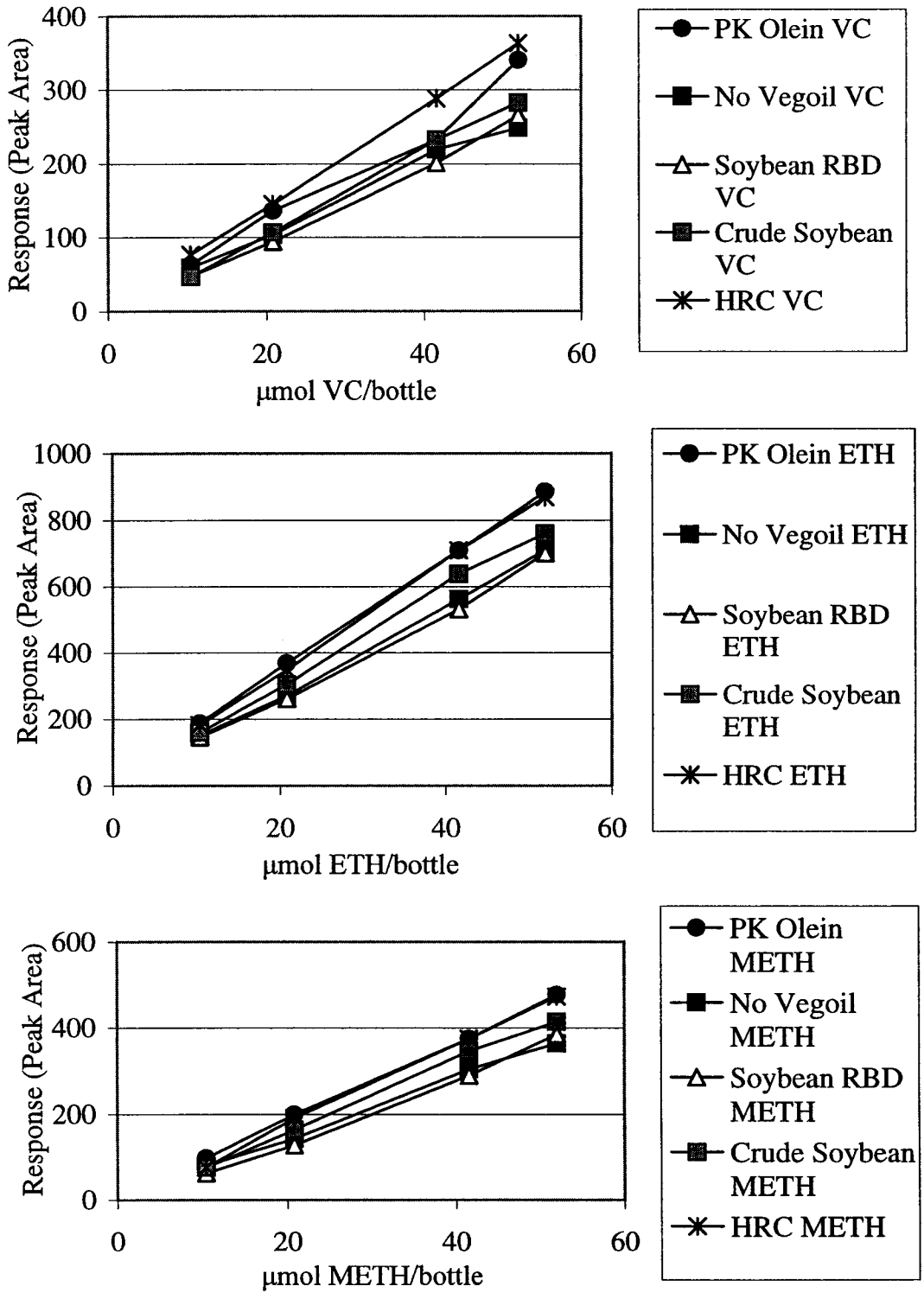

Figure 3.2 Comparison of VC, ETH and METH Calibration Curves for Different Vegoil-Amended Standard Bottles

Figures 3.1 and 3.2 demonstrate the difference in the resulting headspace concentrations as a result of partitioning into the vegoil layer. Headspace concentrations were denoted by peak area responses that were generated by the gas chromatographs. Partitioning into the various vegoils was most evident for PCE. The lesser-chlorinated ethenes were less affected (with effect diminishing as TCE>cis-DCE>VC). ETH and CH_4 were virtually unaffected by the 100 mg oil. Practically speaking, the only compound whose headspace concentration was significantly affected by vegoil was PCE. On the other hand, the slopes of the calibration curves were highest for bottles with HRC®, assumed to be caused by the salting-out effect of HRC®.

Calibration with water and vegoil would have been applied in our analyses if it were not for the fact that vegoil concentration changed with time. As such, the effect of partitioning cannot be accurately estimated in live vegoil-fed cultures. In view of this, calibration with standards with no vegoil was employed. It was understood that with such calibration, PCE concentrations (in particular) in vegoil-fed cultures would be underestimated while vegoil still remained. However, once all vegoil had been degraded, the actual concentration of the chloroethenes would be revealed and accurate accounting of them could be performed.

3.D.4. Volatile Acids Analysis

A Perkin-Elmer Corporation Autosystem gas chromatograph with a 0.53-mm x 15-m Nukol® capillary column (Supelco, Inc.) and an FID was used for analysis of the VFAs acetic, propionic, isobutyric, butyric, valeric, isovaleric, and hexanoic acids [*20*]. The N_2 carrier gas flow rate was 10 mL/min, the injector temperature was 200°C and the detector temperature was 250°C. The flame was maintained with H_2 (30 psi, 45 mL/min) and air (30 psi, 450 mL/min).

For VFA analysis, a 0.5-µL sample was injected onto the column that was held at 90°C for 15 min. The retention times for this run were: acetic acid, 3.3 min; propionic acid, 5.2 min; isobutyric acid, 6.0 min; butyric acid, 7.8 min; valeric acid, 8.4 min; isovaleric, 9.7 min; and hexanoic acid, 12.8 min.

Detector output was integrated by a PE Nelson model 1022 integration system and then analyzed by Turbochrom® software.

Samples of 0.25 or 0.5 mL were removed from reactors or serum bottles via a 3-mL disposable syringe and were immediately filtered through a 0.2-µm PTFE filter (Gelman Sciences) into a 2-mL vial. The samples were acidified by the addition of 8N H_3PO_4 (10 µL per 0.5 mL of sample) to obtain a pH of between 1 and 2, and refrigerated until analysis.

Identification of the volatile acids was performed through comparison of retention times with those of known standards. Volatile acids stock solutions were prepared by adding known gravimetric amounts of neat acids to 1 L of distilled water. Standards were prepared by adding volumes of the stock solutions to a 100-mL volumetric flask. The flask was filled to just below the 100-mL mark with distilled water.

3.D.5. Monitoring of Vegoil Consumption

The status of vegoil consumption was estimated indirectly from the accumulation of methane in the serum bottle. Most of the reducing equivalents of added vegoil ended up routed through methanogenesis. Vegoil was assumed to be completely consumed when methane concentration in bottle headspaces reached asymptotic levels and ceased to increase. We assumed the empirical formula of vegoil to be $C_8H_{16}O$ (regardless of which kind), and the amount of methane expected to arise from 50 mg of vegoil was thus projected to be around 2.25 mmol or 17.97 meq.

3.D.6. Particulate Organic Nitrogen Analysis

Biomass was estimated from the particulate organic nitrogen (PON) content of samples. A microbial cell composition of $C_5H_7O_2N$ was assumed. A 100-mL volume of enrichment culture sample or a basal medium blank was divided into two equal portions. One portion was underwent total-sample Total Kjeldahl Nitrogen (TKN) analysis according to Standard Methods 4500-N_{org} – B. Macro-Kjeldahl Method. The other was filtered through SUPOR-200, 0.2- μm filter (Gelman Sciences) to produce a filtrate. The filtrate was collected and then analyzed through TKN procedures to obtain the soluble nitrogen content of the sample. Particular Organic Nitrogen (PON) was calculated by subtracting the soluble TKN from total-sample TKN. The following calculations were performed to convert from nitrogen content to biomass (volatile suspended solids, VSS):

mg total TKN/L sample =

{[mL titrant for sample] x [0.28 mg-N/mL titrant]/mL sample} x 1000 mL

mg soluble TKN/L sample =

{[mL titrant for filtrate] x [0.28 mg-N/mL titrant]/ mL filtrate} x 1000 mL

mg PON/ L sample =

mg total TKN/L – mg soluble TKN/L

mg biomass VSS ($C_5H_7O_2N$)/L =

[mg particulate organic-N/ L sample] x 113/14

CHAPTER FOUR

EXPERIMENTAL RESULTS

4.A. Presentation of Experimental Data

The data for Palm Kernel Oil, Crude Soybean Oil, Refined Bleached and Deodorized Soybean Oil, Hydrogen Release Compound® and the unfed controls are shown in this chapter and Appendix A. Representative data from one bottle in each set of bottle triplicates are shown in Figures 4.1 to 4.10, while the rest are displayed in Figures A.1 to A.19. Behaviors among triplicates were by-and-large similar. However, one exception was the behavior of triplicate c) of "RBD + amds" which was adversely affected by improper purging of sample (a human error) which caused fatal perturbations to the culture. Consequently, its data are not shown.

Each Figure shows time-course-profiles of a) chloroethenes and b) H_2 and methane. In the Figures for the vegoil samples and the unfed controls (in Figures 4.1 to 4.8), bar charts that account for the total electron equivalents (assuming complete oxidation to CO_2) used in the

production of volatile fatty acids (VFAs), methane and chloroethenes are also included. VFAs and biomass measurements are summarized in Tables 4.2 and 4.3, respectively.

For the sake of convenience, the names of the vegoil samples, unfed controls and HRC® controls are abbreviated as shown in Table 4.1:

Table 4.1 Abbreviations of Sample Names

Sample	Abbreviation
PCE only	"PCE only"
PCE + yeast extract + vitamins	"PCE+amds"
PCE + Palm Kernel Oil	"PK only"
PCE + Palm Kernel Oil + yeast extract + vitamins	"PK + amds"
PCE + Crude Soybean Oil	"Crude only"
PCE + Crude Soybean Oil + yeast extract + vitamins	"Crude+amds"
PCE + Refined Bleach Deodorized Soybean Oil	"RBD only"
PCE + Refined Bleach Deodorized Soybean Oil + yeast extract + vitamins	"RBD + amds"
PCE + HRC®	"HRC® only"
PCE + HRC® + yeast extract + vitamins	"HRC®+amds"

VFA data were graphed by summing up the electron equivalents of the volatile fatty acids (referenced to total oxidation to CO_2) for each sample and plotting the data onto a bar chart. The amount of methane produced in each sample was converted into equivalents per bottle (referenced on a CO_2 basis) and stacked on top of the VFA bars. In addition, the amounts of chloroethenes produced in each sample were also converted into equivalents per bottle (this time, referenced on a PCE basis) and stacked on top of the methane bars. In this way, the total amount of electron equivalents (eeq) of electron donor utilized (excluding eeqs used for biomass growth) could be accounted. The assumed amount of total equivalents supplied by 50 mg of vegoil was 17,970 μeq/bottle. None of the stacked bars exceeded this amount.

4.B. Comparison of the Electron Donors Palm Kernel Oil; Crude Soybean Oil; and Refined, Bleached and Deodorized Soybean Oil

All vegoil-fed bottles and unfed controls were prepared on the same day in the manner stated in Chapter 3. An initial (Day 1) hydrogen level of around 2 x 10^{-4} atm was observed for most samples. This initial amount of hydrogen was likely introduced from the glovebox environment in which the samples were prepared. The hydrogen level rapidly declined to less than 1 x 10^{-5} atm within a day.

Decline in PCE levels in the vegoil samples can be attributed to two factors: a) partitioning into the vegoil layer, and; b) dechlorination to TCE. Decline by partitioning was normally observed immediately after respiking of PCE. This decline appeared as a sharp drop in PCE concentration with no apparent increase in any of the other chloroethenes (TCE, DCE, VC, ETH). Occasionally the PCE concentration would rise again slightly due to the partitioned PCE being released through breakdown of the vegoils. In other cases, the PCE concentration would

continue to fall if the rate of dechlorination exceeded the rate of release from the vegoil.

Decline in PCE levels through degradation could be confirmed by the increase in dechlorination by-products TCE, DCE, VC or ETH. However, mass balance on PCE and dechlorination by-products proved to difficult due to gases escaping through the bottle septum. Large amounts of methane produced by the mixed culture caused high headspace pressures (up to ~2 atm), which, in turn, cause some portion of the gases to escape through the septa.

4.B.1. Performance of Unfed Controls

Dechlorination rates were relatively slow for the unfed controls. It took 30 days for "PCE only" to eliminate the first spike of 11 μmol of PCE (Figure 4.1), while it took 20 days for "PCE + amds" to do the same (Figure 4.2). Maximum methane produced was around 50 to 60 μmol for the two types of unfed controls. The limited reducing equivalents that sustained methane production and chloroethene degradation were probably contributed mostly by decaying biomass. The equivalents contributed by the YE and vitamin amendments were apparently negligible, since methane levels were about the same whether or not

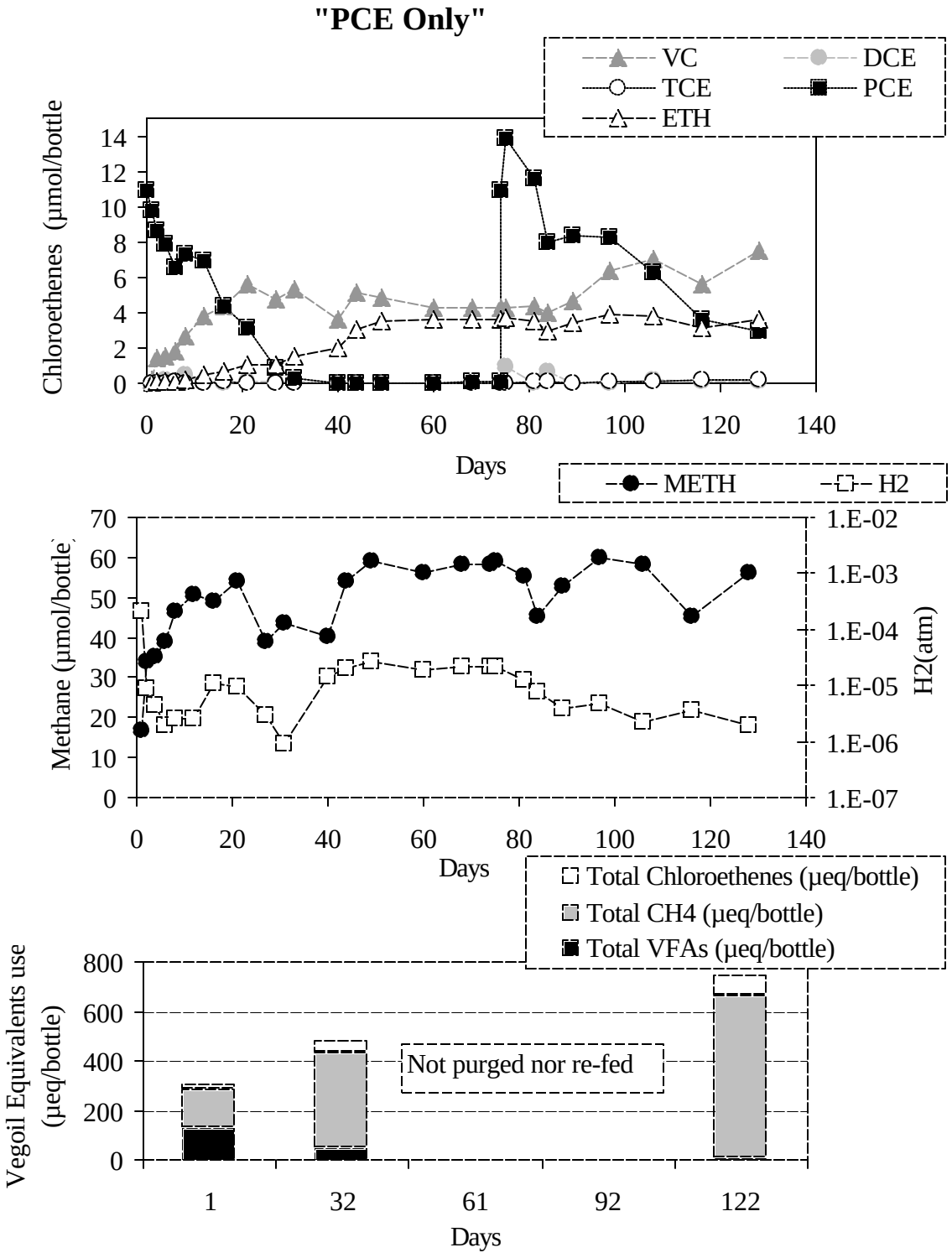

Figure 4.1. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil (without Yeast Extract and Vitamins).

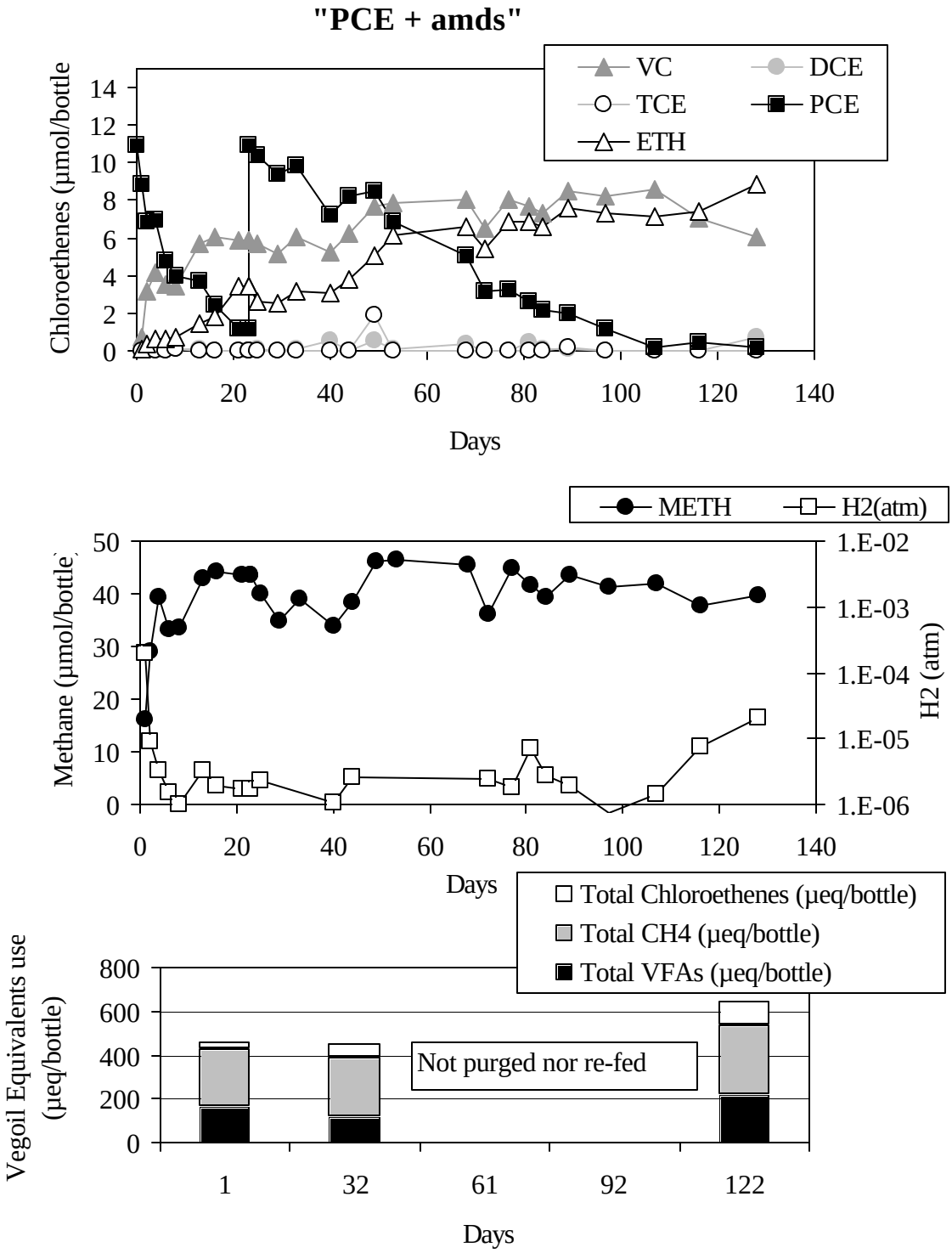

Figure 4.2. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil + Vitamins + Yeast Extract.

amendments were added. However, what may appear negligible to total equivalents in the end may nonetheless have been significant to dechlorination, since relatively few equivalents would be needed to dechlorinate 11μmol (88 μeq) PCE to ETH. "PCE only" was capable of handling one full PCE spike and then ceased to fully dechlorinate the second PCE spike. "PCE + amds" handled two PCE spikes within the 140-day monitoring period and might still have had the potential to dechlorinate further PCE spikes, though dechlorination of the second spike was considerably slower than of the first.

4.B.2. Performance of Palm Kernel Oil Samples (PK only and PK + amds)

In this sub-section the behaviors of the two palm kernel oil-fed samples ("PK only" and "PK + amds") are described in detail (Figures 4.3 and 4.4).

4.B.2.a. Initial Dechlorination Performance of Palm Kernel Oil Samples. The initial PCE spike took "PK only" 10 to 15 days to degrade. In the same period of time, "PK + amds" was able to handle 3 PCE spikes. One may suspect that the superior performance of "PK + amds"

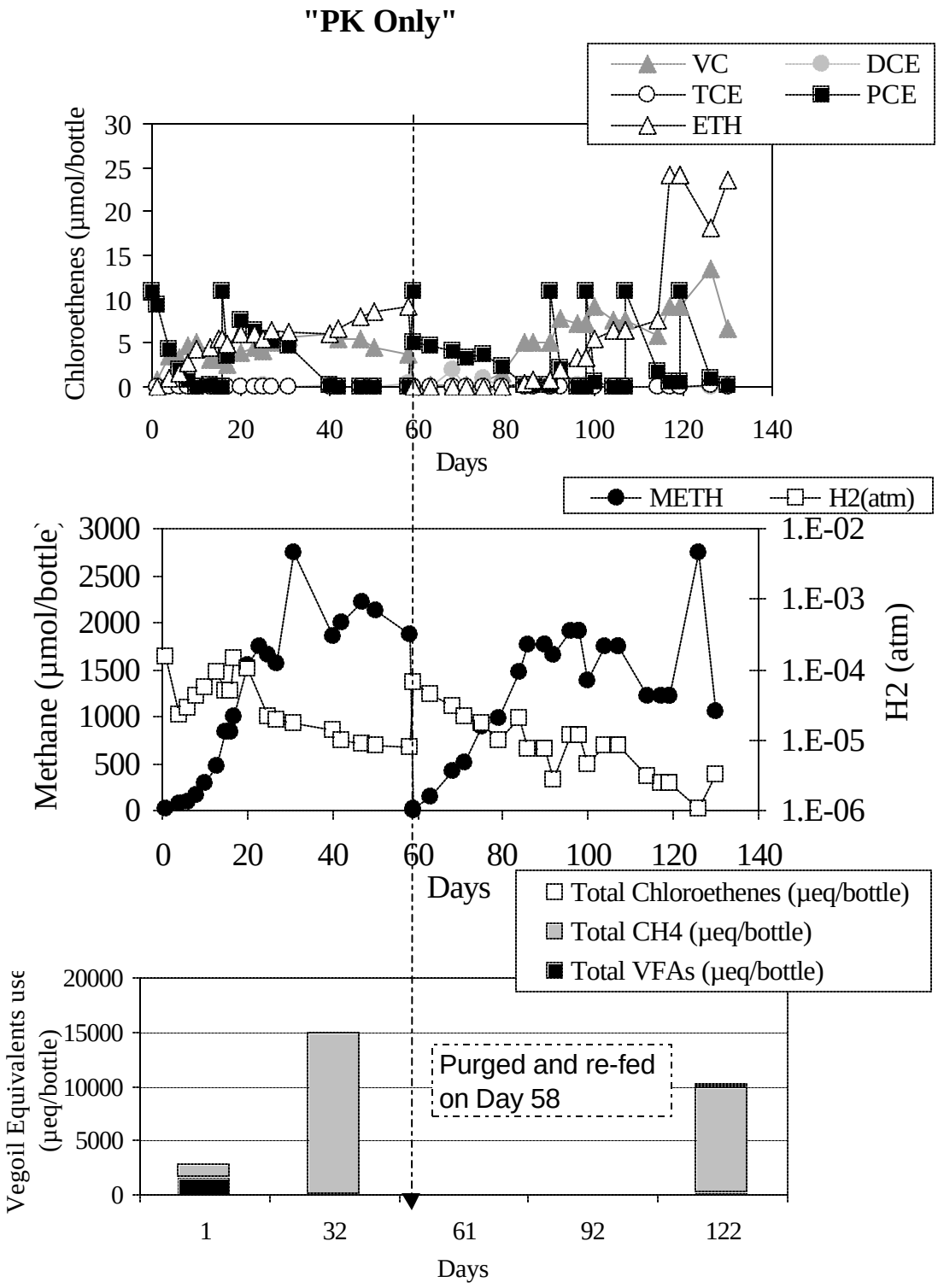

Figure 4.3. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil (without Yeast Extract and Vitamins).

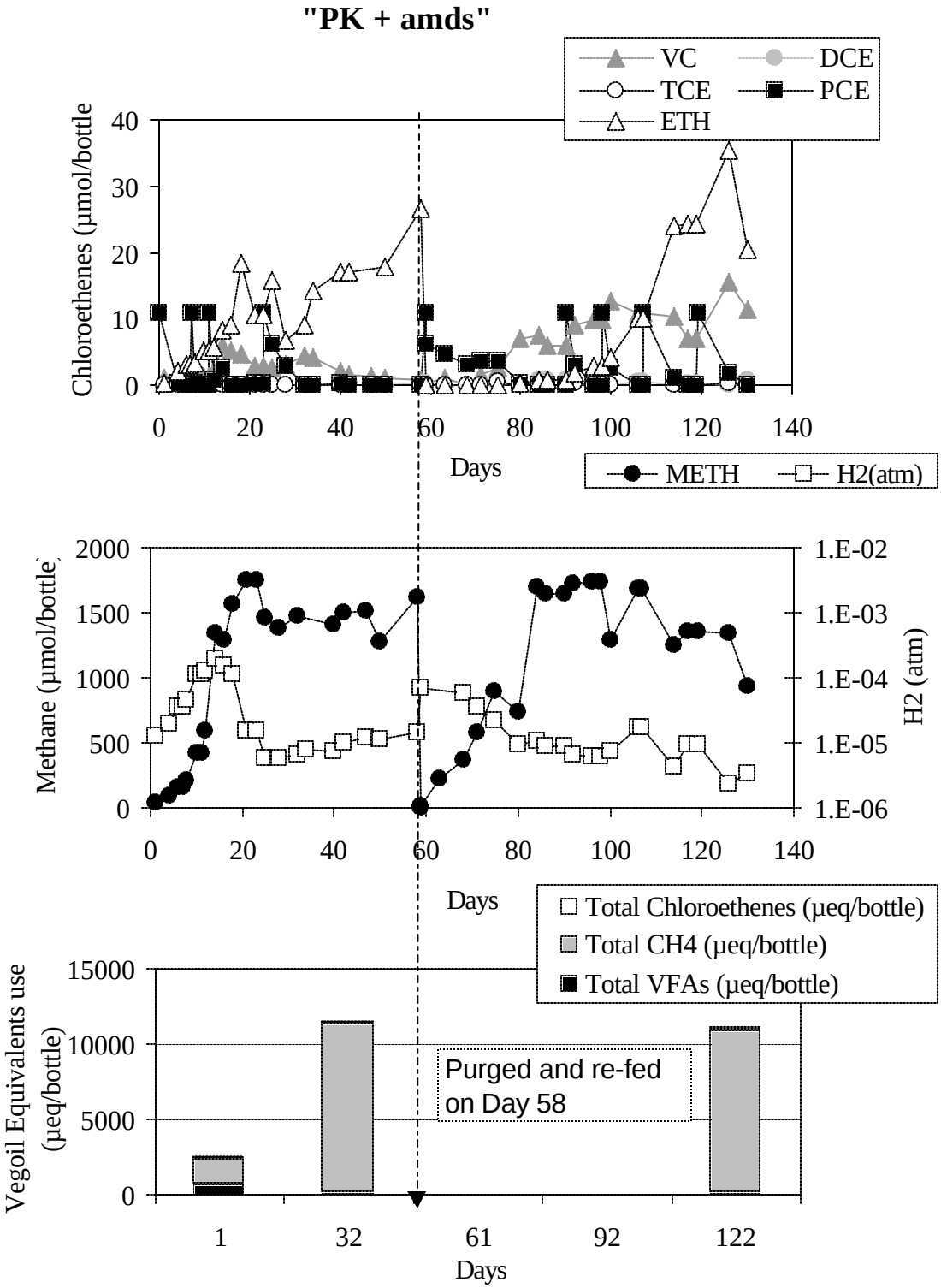

Figure 4.4. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil + Yeast Extract + Vitamins.

was caused by its utilization of yeast extract and vitamins as electron donors instead of the vegoil. After all, in the initial few days, fermenters might still be adapting to utilizing long-chain fatty acids.

However, this is unlikely to be true because the unfed control, "PCE +amds", exhibited much poorer dechlorination performance when compared to "PK + amds". This shows that reliance on yeast extract and vitamins as sole electron donors could not have caused the superior performance in "PK + amds". Rather, it is suggested that the amendments acted as catalysts for the fermentation of Palm Kernel Oil.

4.B.2.b. Time Course Profile of Hydrogen and Methane. Hydrogen peaked around Day 20 at 1.4 x 10^{-4} atm for "PK only" while hydrogen peaked around Day 15 at 1.9 x 10^{-4} atm for "PK + amds". Shortly afterwards, hydrogen levels in both samples began to decline sharply; by Day 25, they had dropped to less than 1 x 10^{-5} atm. Hydrogen levels were maintained at slightly under 1 x 10^{-5} atm until purging and re-feeding on Day 58. Methane concentrations rose until they leveled off around Day 20. Maximum methane concentration ranged from 2000 to 2300 μmol/bottle among "PK only" triplicates and 1600 to 2000 μmol/bottle among "PK + amds" triplicates.

4.B.2.c. Late Dechlorination Performance. Because of oversight on the part of the experimenter, PCE respikes were not carried out in a timely fashion in the period after methane has leveled off (from Day 20 to Day 58). As a result, there were long periods of time when there was no PCE for the culture to degrade. Most serum bottles only received one PCE respike during this period. On the other hand, dechlorination of PCE was very slow. Prior to the leveling-off of methane, 11 μmols of PCE took an average of 18 days for "PK only" to deplete while it took around 5 to 6 days for "PK + amds" to deplete. After the leveling-off of methane, the average time taken to handle 11 μmols of PCE was 18 days for "PK + amds". It is projected that if the PCE spiking regime were more faithfully adhered to, the cultures would have handled one or two more PCE respikes. However, because the vegoil was already depleted in the period where methane had leveled-off, dechlorination in the latter period was less of a testament to the electron donor ability of vegoil than that of decaying biomass.

4.B.2.d. VFA measurements. VFA measurements on Day 8 revealed a substantial amount of VFAs in the Palm Kernel Oil samples (Table 4.2). Among the various VFAs, acetate was the predominant species. Concentration of acetate was 1000 μmol/bottle for "PK only"

Table 4.2. Summary of Volatile Fatty Acids Measurements on Day 8, 40 and 128

	Day	Acetic µmol/L	Propionic µmol/L	Isobutyric µmol/L	Butyric µmol/L	Isovaleric µmol/L	Valeric µmol/L	Hexanoic µmol/L	Total ueq/bottle of VFA
No Vegoil	8	83.05			46.92	0.61		0.25	161.08
	40		21.99		40.47				74.49
	128	699.20	11.36	0.35	17.98	9.86	0.30	0.66	226.87
No Vegoil	8	60.72			23.80	4.39			103.79
+ Yeast Extract	40	83.46	34.83	0.13	34.24		0.13	0.11	107.31
+ Vitamins	128	493.47	4.48	0.20	16.26		0.35	0.59	433.14
Palm Kernal Oil	8	1538.22			30.08	4.24			1301.77
	40				54.50			0.41	73.54
	128	161.38	0.79	2.23	64.30		0.13	0.04	218.44
Palm Kernal Oil	8	631.63	1.61		32.57	5.58			580.88
+ Yeast Extract	40				64.71			0.99	88.40
+ Vitamins	128	72.64	5.40	2.67	10.13	0.14	0.83	0.08	79.65
Crude Soybean Oil	8	430.30			9.77	1.38			359.67
	40				32.25			0.42	21.95
	128	410.66	21.08	1.15	42.01	23.75		0.20	306.65
Crude Soybean Oil	8	172.04	1.13		1.59				93.35
+ Yeast Extract	40		45.15	4.16	65.52		4.16		98.59
+ Vitamins	128		28.57			18.29	0.06	25.36	56.28
Refined Bleached and	8				7.88	1.41			18.19
Deoderized Soybean Oil	40		5.31		27.55			0.32	24.01
	128	1261.46	50.21		15.88	23.36		0.44	478.93
Refined Bleached and	8	144.25				1.74			116.91
Deoderized Soybean Oil	40	554.18							147.78
+ Yeast Extract + Vitamins	128	1359.32	8.43	0.30		14.39		7.26	410.99

and 700 μmol/bottle for "PK + amds". These concentrations are very much higher than the half-velocity constant (K_s = 10 mg/L) for acetotrophic methanogenesis. In the period after Day 8, methanogenic activity began to accelerate dramatically.

4.B.2.e. Behavior after Purging and Re-feeding with Vegoil.

Methane levels in "PK only" and "PK + amds" leveled-off in the period after Day 30 and Day 21, respectively. This is taken as a indication of complete consumption of vegoil. Consequently, as part of the protocol described in chapter 3, "PK only" and "PK + amds" were purged free of chloroethenes and methane and then re-fed with Palm Kernel Oil on Day 58. Behaviors of "PK only" and "PK + amds" were very similar in this period. Perturbations during the "purge and re-feed" process probably affected the subsequent dechlorinating performance of the culture. It was observed that the degradation of the following PCE spike after purge and re-feed was relatively slow and took an average of 25 days to deplete for both samples. A hydrogen peak can be observed almost immediately in each of the bottles after they were re-fed with vegoil. However, the magnitude of this second peak (around 4.5 x 10^{-5} atm for "PK only" and 6.5 x 10^{-5} atm for "PK + amds) was generally smaller than the one from

the initial vegoil feed (1.4 to 1.9 x 10^{-4} atm for both "). A possible explanation for this was that the increase in methanogenic population probably depleted the second hydrogen peak faster than with the first hydrogen. The reduction in time lag between the feeding of vegoil and the occurrence of the hydrogen peak can be attributed to the fermenters being already acclimated to using vegoil as a substrate by the second feed.

Meanwhile, methane levels rose dramatically and reached maximums of 1500 to 1700 μmol/bottle for both samples within 30 days after re-feeding.

Interestingly, dechlorination performance improved a great deal in the period after the methane had leveled off (Day 90 onwards). Four spikes of PCE were introduced to each of the "PK + only" and "PK+ amds" triplicates during this period and were degraded within 10 days per spike on average. Since it has been reasoned that vegoil had already been depleted by this period, the remaining electron donor source was presumed to be the decaying biomass. However, the rate of dechlorination contrasted sharply against that of the corresponding period of the first feed of vegoil. Dechlorination in the corresponding period in the first feed took 18 days per spike while in the second feed it took 10 days per spike. This difference is likely to be the result of dechlorinators

recovering from the perturbations of "purging and re-feeding". Similar performance can be found in samples fed with other vegoils that were not perturbed as much as in the palm kernel oil samples. Although vegoil was depleted in the mentioned period, the sheer amount of accumulated decaying biomass probably produced sufficient hydrogen to drive the rapid dechlorination of the PCE peaks.

Biomass data in Table 4.3 supports this hypothesis. Table 4.3 shows that total biomass in the vegoil cultures on Day 140. Biomass in the unfed controls were 50 mg/L for "PCE only" and 90 mg/L for "PCE + amds", while those in the vegoil samples ranged from 130 mg/L to 180 mg/L. The theoretical yield of vegoil-fed methanogenic biomass is calculated to be 0.252 mg $C_5H_7O_2N$/meq (see Appendix B) and this translates to a 9.06 mg increase in biomass per 100 mg vegoil. This gives a final theoretical biomass of 161.4 mg/bottle which is very close to the average measured biomass of the vegoil-fed bottles of 157.6 mg/L. One thing to note is the fact that these biomass values were measured at the end of the 140-day period when the samples have been starved for at least 50 days. Hence, one can imagine the sheer mass of microroganisms prior to the depletion of vegoil and the possibility that the endogenous decay of

Table 4.3 Summary of Total Kjeldahl Nitrogen and Biomass Data

	Total Kjeldahl Nitrogen mmol-N/L	Soluble Organic Nitrogen mol-N/L	Particulate Organic Nitrogen mol-N/L	**Biomass * (mg VSS/bottle)**
Basal Media	3.26	3.11	0.15	
PCE Only	5.33	4.73	0.60	**5.13**
PCE + amds	5.95	5.00	0.95	**9.03**
PK	5.33	4.06	1.28	**12.75**
PK + amds	5.49	3.87	1.62	**16.62**
Crude	5.96	4.20	1.76	**18.25**
Crude+amds	5.46	3.91	1.55	**15.86**
RBD	5.23	3.95	1.27	**12.72**
RBD + amds	5.84	4.07	1.77	**18.34**

** Biomasses were calculated by assuming the empirical formula of* $C_5H_7O_2N$

such biomass could sufficiently fuel the dechlorination reaction of the culture.

4.B.3. Performance of Crude Soybean Oil Samples ("Crude only" and "Crude + amds")

Crude Soybean Oil samples exhibited behavior similar to that of Palm Kernel Oil. As expected, dechlorination performance differed in the periods before and after methane had leveled off. In the first feed of crude soybean oil to "Crude only", average degradation time for 11 μmols of PCE in the period before methane leveled-off was 24 days while degradation after required 30 days. For "Crude +amds" it took 14 days and 31 days respectively. Some TCE was mistakenly introduced into some of the triplicates of "Crude only" and "Crude + amds" on Day 44 but was degraded normally with no adverse effects observed on the culture. The maximum amount of methane produced was 1900 μmol/bottle for both "Crude only" and "Crude + amds".

The purge and re-feed process was carried out on Day 96 for both "Crude only" and "Crude + amds". Less perturbations were affected on the culture during the purge and re-feed process because of better experience on the part of the experimenter. Contrary to what happened in

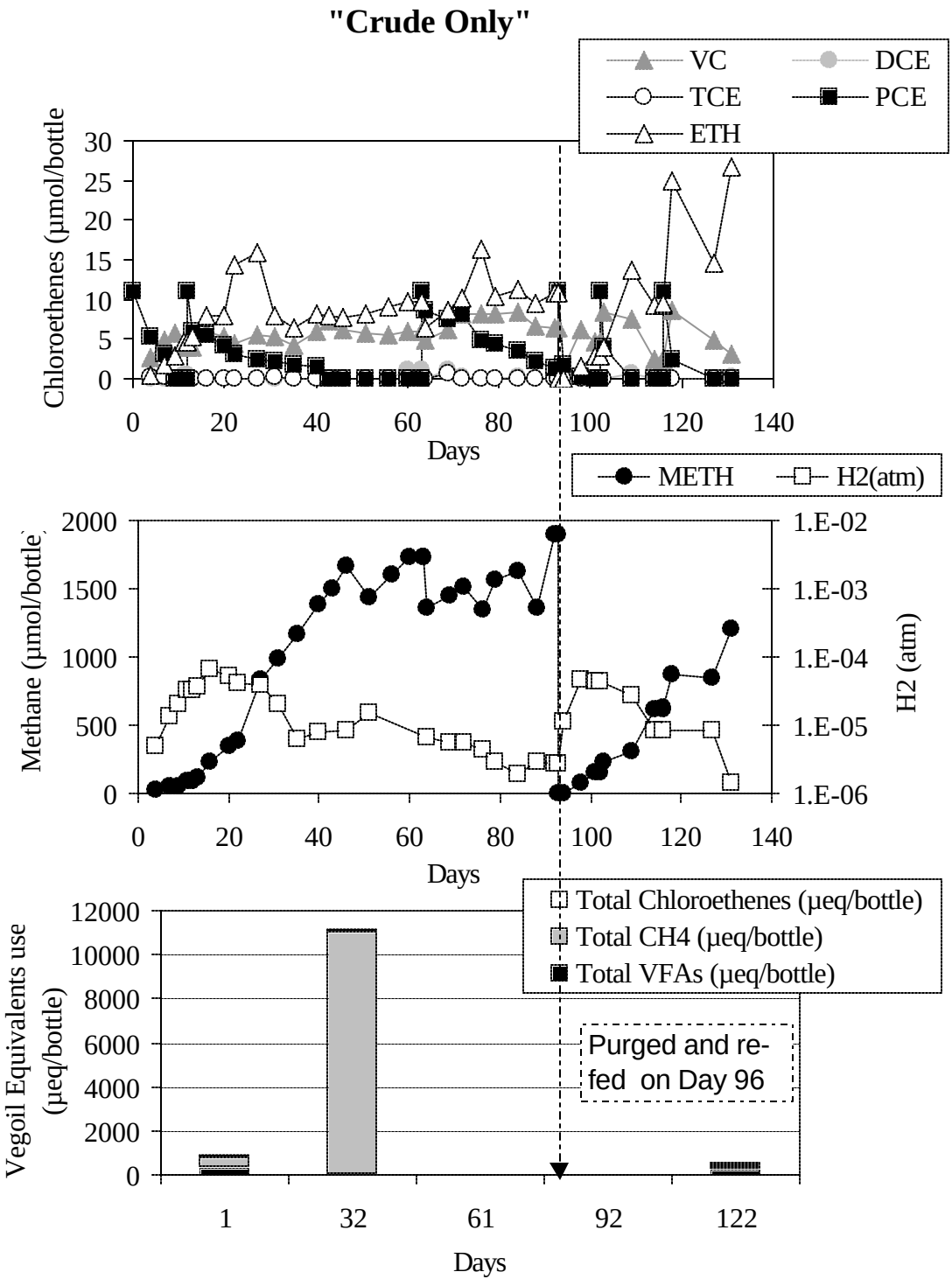

Figure 4.5. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil (without Yeast Extract and Vitamins).

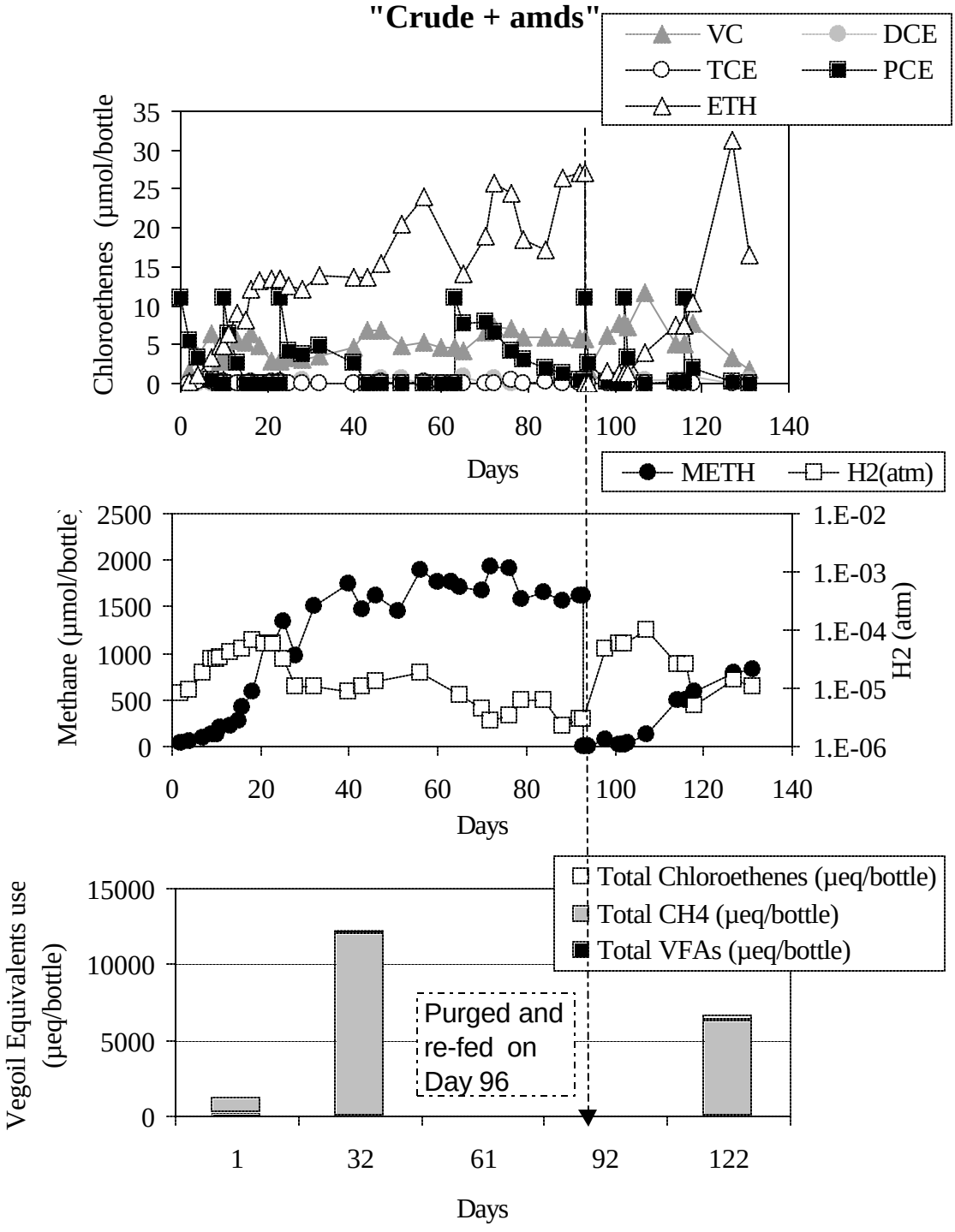

Figure 4.6. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil + Yeast Extract + Vitamins.

the Palm Kernel Oil samples after purge and re-feed, dechlorination went well in the period before methane leveled-off. Average time to degrade one PCE peak within this period took about 11 days for both "Crude only" and "Crude + amds". Unfortunately, the 140-day period did not allow the monitoring of dechlorination behavior after methane had leveled-off. It is projected that biomass in the culture would be able to sustain rapid dechlorination just as in the palm kernel oil samples.

4.B.4. Performance of Refined Bleached and Deodorized Soybean Oil Samples ("RBD only" and "RBD + amds")

Refined Bleached and Deodorized Soybean Oil samples also shared similar behavior to that of Palm Kernel Oil. In the first feed of crude soybean oil to the samples, average degradation time for 11 μmols of PCE in the period before and after methane leveled-off was 21 and 31 days respectively. For "RBD + amds" it took 9 days and 27 days respectively. Some TCE was mistakenly introduced into some of the triplicates in the "RBD only" and "RBD + amds" on Day 44 but was degraded normally with no adverse effects observed on the culture. The maximum methane level was 1700 μmol/bottle in both "RBD only" and "RBD+amds".

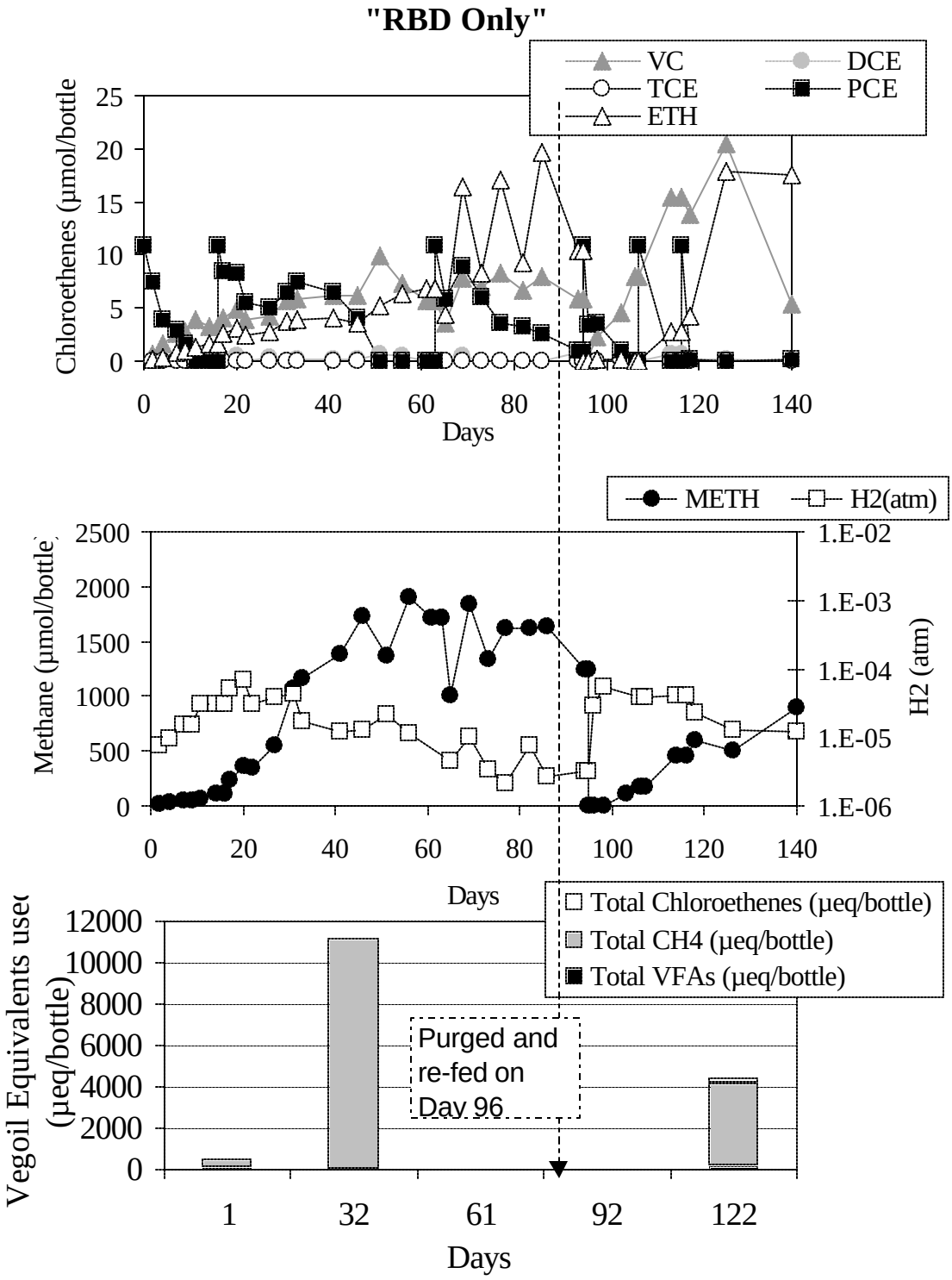

Figure 4.7. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Refined Bleached and Deodorized Soybean Oil (without Yeast Extract and Vitamins)

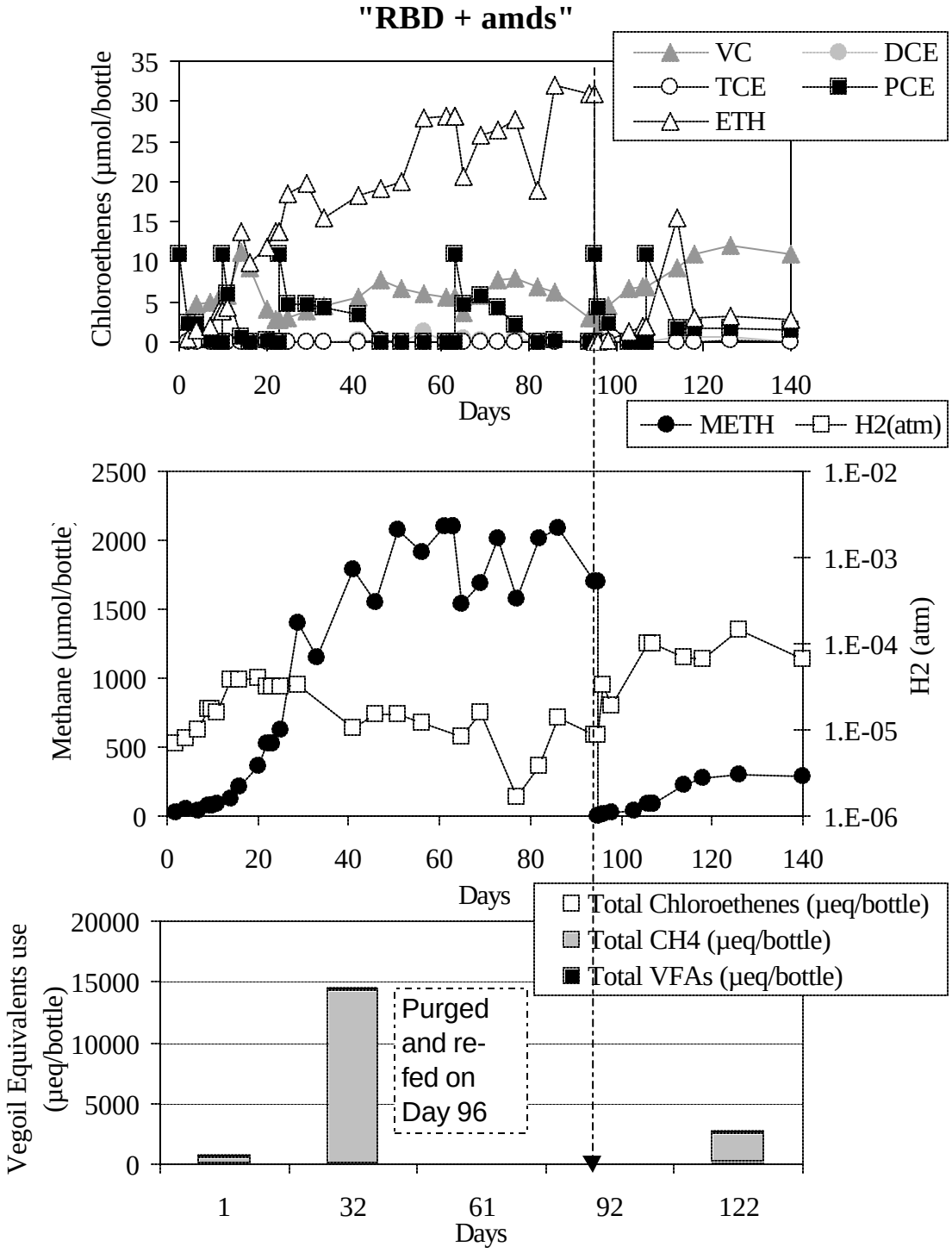

Figure 4.8. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Refined Bleached and Deodorized Soybean Oil + Yeast Extract + Vitamins.

The purge and re-feed process was carried out on Day 96 for both "RBD only" and "RBD + amds". Less perturbations were affected on the culture during the purge and re-feed process because of better experience on the part of the experimenter. Contrary to the Palm Kernel Oil samples, after purge and re-feed dechlorination went well in the period before methane leveled-off. Average time to degrade one PCE peak within this period about 11 days for both "RBD only" and "RBD + amds". The 140-day period did not allow the monitoring of dechlorination behavior after methane had level off. However, it is projected that biomass in the culture would be able to sustain rapid dechlorination just as in the palm kernel oil samples.

4.B.5. Performance of Hydrogen Release Compound® Samples ("HRC® only" and "HRC®+ amds")

The purpose of monitoring HRC®-fed samples was to assess its ability to sustain dechlorination and to use it as a benchmark against the other vegoils. Because commercial literature [*43*] proclaimed it as a viable and effective donor, it was also used as controls to assess the health of the source culture. Once HRC® culture was observed to be dechlorinating normally, the source culture was concluded as healthy and

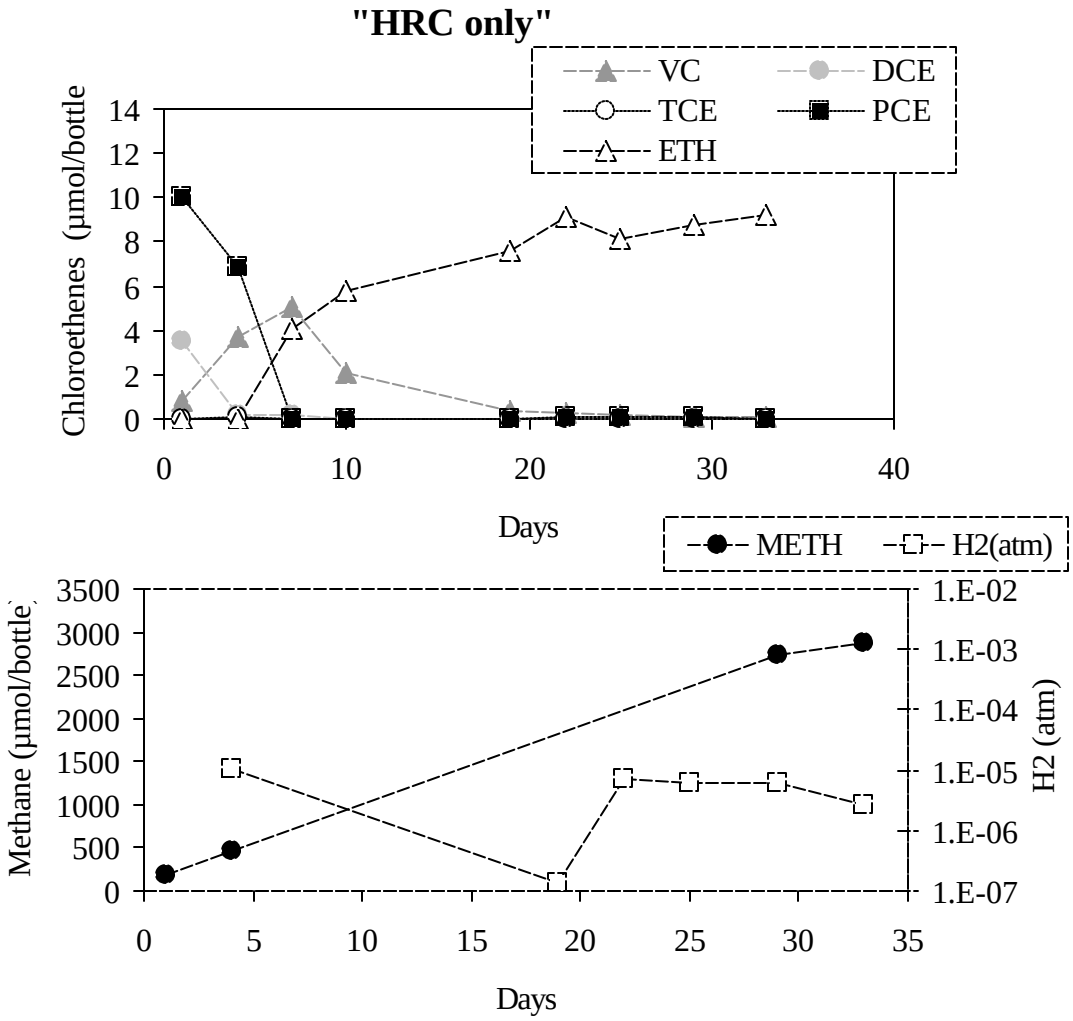

Figure 4.9. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) (without Yeast Extract and Vitamins).

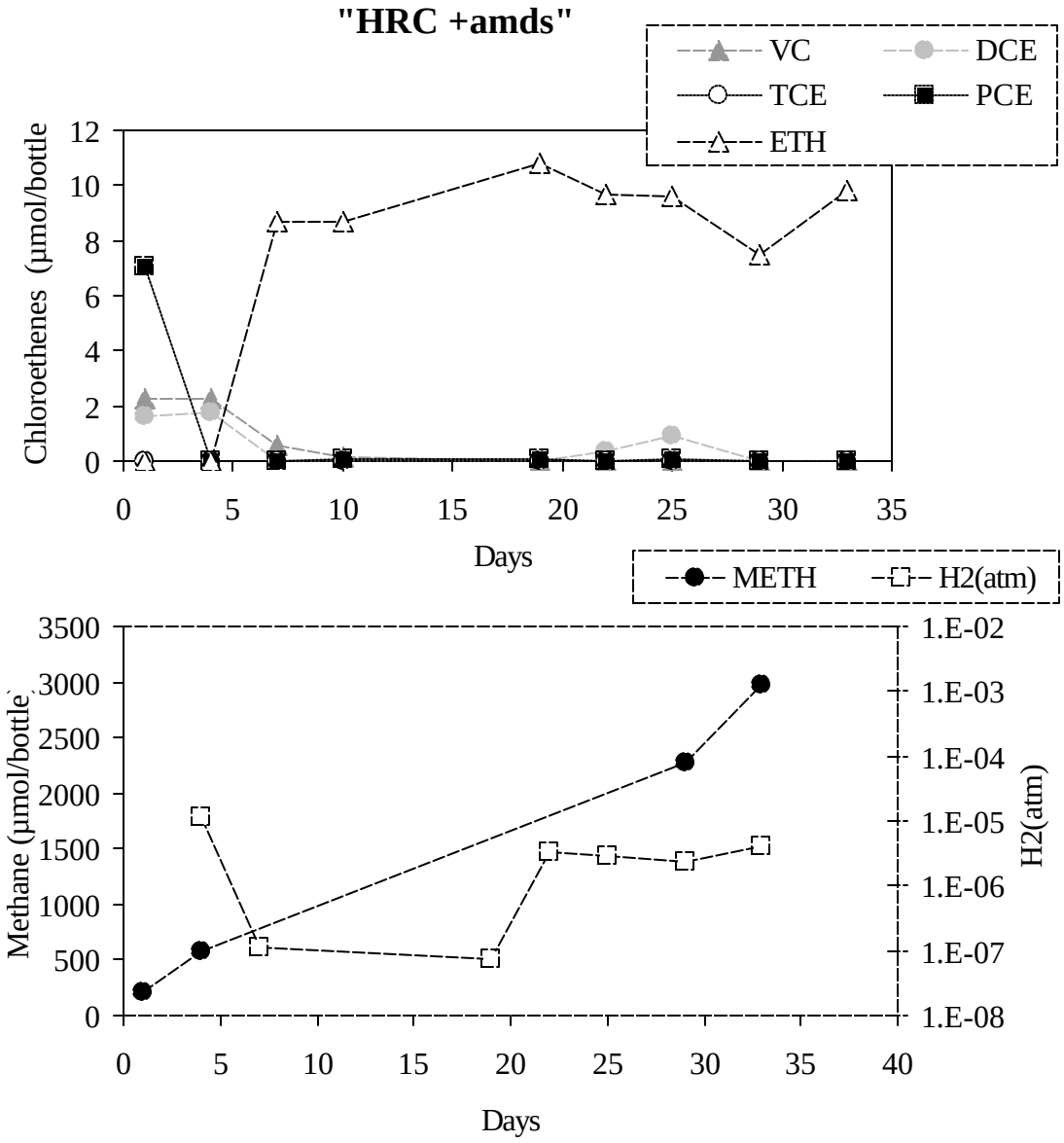

Figure 4.10. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) + without Yeast Extract + Vitamins.

monitoring of HRC® culture was stopped. One point to note is that 100 mg of HRC® was fed to the culture and expected maximum methane produced was 2027 μmols.

Only one PCE spike of 11 μmol was introduced into the HRC culture. It was rapidly depleted within 7 days for "HRC only" and 5 days for "HRC + amds". Total conversion to ethene was accomplished around 20 days for both samples. Therefore the source culture was determined to be healthy.

Throughout the monitoring period of 40 days, methane increased in a linear fashion and did not level-off in the same fashion seen in the vegoil cultures. This indicated an excess supply of hydrogen in the culture. However, hydrogen levels were maintained at levels lower than that shown in the vegoils samples, and thus, by theory, competitive advantage was given to the dechlorinators. Hydrogen profiles demonstrated a considerable decrease in the hydrogen level while PCE was being degraded, thus indicating a preference of electron donor towards dechlorination.

4.B.6. Total Kjeldahl Nitrogen and Biomass Measurements

Because of the destructive nature of the Total Kjeldahl Nitrogen (TKN) tests and the relatively large amount of sample required, the measurements of organic nitrogen were carried out only at the end of the monitoring period (on Day 140). The procedure is described in Chapter Three and a summary of the results can be found in Table 4.3. The biomass in vegoil bottlee ranged from 13 to 18 mg/bottle. All samples contained at least 3.8 mg-N/L of soluble organic nitrogen. This shows that the basal salts medium contained sufficient nitrogen and no nitrogen limitation was experienced in any of the samples. The amount of biomass in the unfed controls were 5.13 mg/bottle for "PCE Only" and 9.03 mg/bottle for "PCE + amds". Biomass concentration of the low PCE/butyrate culture was around 6.82 mg/bottle according to Fennell [*20*]. If we assume the initial biomass in the culture was 6 mg/L, an overall yield (ignoring the effects of endogenous decay) was about 7 to 12 mg VSS per 100 mg of vegoil from two separate feeds. These observations agree with bioenergetic results shown in Appendix B.

4.B.7. Volatile Fatty Acids Measurements.

VFA measurements were performed on days 8, 40, 128. The goal of monitoring of VFAs was to ensure that certain toxic VFAs such as propionic acid do not accumulate to levels harmful to the culture. In addition, it was to ensure that VFAs do not lower the culture pH to damaging levels.

Table 4.2 summarizes measurements of Volatile Fatty Acids on Day 8, 40 and 128. Predominant VFAs were acetic acid, followed by butyric and propionic acid. Although acetic acid levels climbed as high as 1500 umol/bottle in certain bottles, pH of the various culture remained in the 7-8 range throughout the experiment. This demonstrates that the alkalinity of the basal medium was able to handle the acidity of the VFAs.

Virtually no fatty acids longer than 4 carbons were detected. Previously it was assumed that persistent long-chain fatty acids would be formed by the breakdown of vegoil which would then be dissolved into the culture and provide a slow continuous source of hydrogen. However, since experimental data illustrates the lack of such acids, it is hypothesized that the long-chain fatty acids were rapidly broken down to acetate once they were formed. This is consistent with the β-oxidation

model – in which long chain fatty acids are broken down two carbons at a time and thus acetate is evolved.

CHAPTER FIVE

DISCUSSION

5.A. Dechlorination Performance

The various vegoil-fed cultures tested in this experiment demonstrated the ability to dechlorinate multiple PCE spikes to VC and ETH. In addition, they were able to maintain their dechlorination abilities after purging and re-feeding of culture.

5.A.1. Comparison between Nutrient-Amended Cultures and Non-Nutrient-Amended Cultures

Comparison of the chloroethene profiles of cultures with nutrient amendments (yeast extract and vitamins) and those without tended to yield the conclusion that the former stimulates dechlorination slightly faster than the latter. In the initial few PCE spikes after the feeding of vegoil, “PK + amds”, “Crude + amds” and “RBD + amds” bottles generally exhibited shorter times of dechlorination than “PK only”,

"Crude only" and "RBD only". However subsequent PCE spikes over the long term took similar times to degrade.

An explanation of this phenomenon could rest upon the possible role of nutrient amendments as catalysts in the fermentation from substrate to hydrogen (see Chapter 4). It could be observed from the hydrogen profiles of the various cultures that cultures with nutrient amendments tend to have higher and earlier initial hydrogen peaks than those without. It is likely that this phenomenon caused earlier stimulation of dechlorination and speedier degradation of PCE. As hydrogen levels dropped down to less than 1×10^{-5} atm after the initial peak, the various vegoil cultures basically exhibited similar degradation times.

Both nutrient-amended and non-nutrient amended samples demonstrated the ability to handle multiple PCE spikes under long-term operation without significant deterioration in performance. PCE degradation was carried out to VC and ETH with very little accumulation of TCE and DCE (the large increases in TCE observed in some of the triplicates were caused by TCE spikes mistakenly introduced by the experimenter). This seems to hint that necessary nutrients in vitamins and yeast extract amendments were already present in (or formed from) the vegoils or the inoculum. Longer periods of monitoring would be needed

the experiment was taking place, calibration factors derived from this sort of calibration could not be properly applied. As a result, calibration was performed using standards containing water only. It was understood that chloroethene concentrations (especially PCE) would be somewhat underestimated while fermentation of vegoil was taking place. However, once vegoil had been completely degraded, all partitioned chloroethenes would be released and the measured concentrations should accurately reflect the actual concentrations in the bottles.

The partitioning phenomenon is a unique characteristic of vegoil that could have interesting applications in the field. It was mentioned in the background of this thesis (chapter 2) that partitioning of chloroethenes into the vegoil plume from the contaminant plume could physically lower the plume's downstream concentration. Furthermore, the partitioned chloroethenes and the vegoil layer could form an efficient dechlorination zone because of the abundance of electron donors within the layer. This will be discussed in detail in Chapter 6 (Engineering Significance).

5.C. Competition between Dechlorination and Methanogenesis

The effect of methanogenic competition on dechlorination can be observed from the time course profiles of methane and chloroethenes. The amount of methane generated from each vegoil feed (50 mg) ranged from 1600 to 1900 μmol/bottle. When converted to electron equivalents (oxidation to CO_2 basis), methane accounted for 71% to 85% of the available 17,970 μeqs of vegoil (based on the empirical formula of $C_8H_{16}O$). Because of the loss of gases through the septum due to the high pressure in the bottle headspace (~2 atm) the actual amount of methane produced was probably higher than measured. For the vegoil samples, dechlorination only accounted for 1 to 2% of the total electron equivalence (oxidation to CO_2 basis). Of course, such a percentage is partly attributable to human error, for more PCE would be dechlorinated if PCE respikes were carried out in a more timely manner, However, this would only increase the amount degraded by an extra 0.5% at the very most.

Based on casual examination of these results, vegoil would seem to provide less competitive advantage towards dechlorination than other electron donors such as propionate, butyrate, lactate and ethanol, which

in order to confirm that micronutrients in vegoil (or formed from its fermentation) could indeed perform the task of yeast extract and vitamins.

5.B. Partitioning of Chloroethenes into Vegoil Layer

Apart from dechlorination, decline in measured PCE concentrations can also be attributed to partitioning into the vegoil layer. Decline by partitioning was normally observed in the first few PCE spikes after vegoil was fed. This decline would appear as a sharp drop in PCE concentration with no corresponding increase in any of the other chloroethenes (TCE, DCE, VC, ETH). Occasionally the PCE concentration would rise again slightly due to the partitioned PCE being released by the breakdown of the vegoils. In other cases, the PCE concentration would continue to fall if the rate of dechlorination exceeded the rate of release from the vegoil.

Efforts to take into account the partitioning effect of vegoil were attempted by calibration with standard serum bottles containing both vegoil and water. Unfortunately, since no direct and accurate method was available to monitor the amount of vegoil left in the sample bottles while

were used in microcosm studies by Fennell [*20*]. According to Fennell, the percentages of reducing equivalents (based on fermentation to H_2 basis) channeled towards dechlorination in the above-mentioned electron donors were almost stoichiometric —52%, 50.7%, 23% and 38.2% for propionic-acid-, butyric-acid-, lactic-acid, and ethanol-fed cultures (2:1 donor:PCE ratio) respectively [*20*]. However, to judge that vegoil was a less efficient electron donor than propionate, etc., based on these results would be to overlook several factors in the experiment setups:

Firstly, vegoil was added in large amounts to the serum bottles. The nominal donor:PCE ratio (calculated on a CO_2 basis) for the vegoil cultures was 204:1 initially at each feeding of vegoil. This resulted in an initial donor:PCE ratio that was four to twenty times higher than that in the source culture (10:1).

It has been observed by Fennell [*20*] that the higher the donor:PCE ratio, the faster the dechlorination rate but the lower the donor efficiency. This relation is true regardless of the hydrogen level sustained by the fermentable substrate and is self-explanatory because excess reducing equivalents can only be channeled towards methanogenesis. Given the high donor:PCE ratio in the vegoil cultures, it was not surprising that methanogenesis was overwhelmingly favored.

Of course, not all the donor was available to the culture simultaneously due to the low solubility of vegoil. In fact, the slow dissolution of vegoil was intended as a mechanism for controlling the rate of substrate feeding. However, given the favorable experimental conditions — 35^{o}C incubation temperature and orbital agitation — much of the vegoil was dissolved and rapidly fermented to VFAs (largely acetate) and H_2 within a short period of time. This is verified by the significant initial hydrogen peaks observed in the various vegoil cultures. Hence the retarding effect of low solubility on substrate availability had been negated by the experimental setup.

In the field, such conducive conditions present in the experimental setup may not at all be present. Generally, lower temperatures, less agitation as well as lower populations of fermenters in the field may cause hydrogen production to proceed at a slower and more gradual rate. Methane production under such circumstances would also likely to be more gradual and less abrupt. In such a situation, it is projected that dechlorination might enjoy more competitive advantage over methanogenesis. Still, treatability studies that are designed to reflect vegoil's behavior under field conditions would be needed in order to prove the technology's suitability for a given field.

5.D. Biomass as Electron Donor

Biomass growth was considerable due to the large amount of carbon source in the culture. Methanogenic biomass in particular, was thought to have accumulated quickly due to the favorable conditions towards methanogenesis. Experimental data had also shown good dechlorination performance when vegoil was assumed to be depleted and biomass was suspected to be the predominant source of hydrogen.

Based on the low hydrogen levels (around 1 x 10^{-5} atm) and low methane production rate that subsisted during periods of starvation (i.e. when methane leveled-off), it is likely that biomass could act as a good slow fermenting source that favors dechlorination over methanogenesis. However, the concept of feeding cultures with excess vegoil to produce a pool of slow fermenting biomass would seem to be a rather inefficient method. This is because, as stated earlier, about 71% to 85 % of the reducing equivalents of vegoil is channeled toward methanogenesis. Besides, anaerobic bacteria are generally low-yield cultures: based on bioenergetic calculations, the theoretical yield was 9 mg biomass/100 mg

vegoil. The average observed yield based on experimental results was around 5 mg biomass/ 100 mg vegoil. Therefore the method of growing a culture up to the desired amount that would produce sufficient hydrogen is likely to be both inefficient and time-consuming.

5.E. Volatile Fatty Acids as Electron Donor

Virtually no fatty acids longer than 4 carbons were detected from the VFA measurements. Previously it was assumed that persistent long-chain fatty acids would be formed by the breakdown of vegoil which would then be dissolved into the culture and provide a slow continuous source of hydrogen. However, since experimental data illustrates the lack of such acids, it is hypothesized that the long-chain fatty acids were rapidly broken down to acetate by the process of β-oxidation.

The lack of persistent long-chain fatty acids shows that reducing equivalents were supplied predominantly by the fermenting vegoil. This indicates that the dechlorination zone within the cultures was limited to the vegoil layer. In the field, a limited dechlorination zone would mean

less contact between the contaminant plume and the electron donor. As a result, treatment effectiveness would be vastly reduced.

5.F. Inhibitory Compounds in Vegoil and Its Fermentation By-Products

In the various vegoil- and HRC®-fed cultures, PCE was dechlorinated to VC and ETH with very little accumulation of by-products (TCE and cis-DCE) in between. One can deduce that it was unlikely that vegoils used in the experiments nor any of its breakdown products had inhibitory effects on the dechlorination pathway at the levels employed in these experiments.

One of the objectives of the experiment was to compare the performance of crude soybean oil and refined, bleached and deodorized soybean oil. Crude soybean oil is the cheaper substrate but it contained more "impurities" that may be either beneficial or inhibitory to the culture. Comparison of experimental data from crude soybean oil and refined, bleached and deodorized soybean oil yielded by-and large similar results: Both vegoils were able to dechlorinate PCE successfully to VC

and ETH with similar production of methane. Therefore the "impurities" in crude soybean oil neither exhibited any significant inhibitory or beneficial effects on dechlorination. Field application-wise, crude soybean oil may turn out to be the more economical alternative.

CHAPTER SIX

CONCLUSIONS

The following conclusions can be drawn from this study of vegoil as a hydrogen donor for stimulating anaerobic reductive dechlorination of PCE:

(1) Based on hydrogen profile, vegoil fermented very quickly upon addition – contrary to initial expectations. Rapid fermentation resulted in hydrogen peaks rose to 1.4×10^{-4} atm to 1.9×10^{-4} atm some fifteen to twenty days after addition, after which hydrogen levels declined to less than 1×10^{-5} atm within the subsequent 10 days. Although this may be attributable to the highly conducive experimental conditions, its occurrence at least shows that potential for fast fermentation exists and thus the technology may not be suitable for certain site conditions. Careful treatability studies may be needed in order to implement vegoil technology in the field. Otherwise, the physical or chemical properties of vegoil may need to be modified and new strategies would be needed for implementing vegoil. This will

be discussed in depth in Chapter Seven (Engineering Significance).

(2) Based on methane profile, most of the electron equivalents were channeled towards methanogenesis. Less than 2% of the total electron equivalents (based on oxidation to CO_2) were used in dechlorination. Previous research has shown that the higher the donor:PCE ratio, the faster the dechlorination rate but the lower the donor efficiency. This is true regardless of whether the donor ferments slowly or quickly because excess reducing equivalents can only be channeled towards methanogenesis. Originally, it was thought that the low-solubility (and hence slow dissolution) of vegoil could act as a mechanism for regulating the donor availability of vegoil. However, since the significant methanogenesis observed disproved this point, other strategies for implementing vegoil may be needed.

(3) Persistent long-chain fatty acids were not detected. Virtually no fatty acids longer than 4 carbons were found from the VFA measurements. It is hypothesized that the long-chain fatty acids were rapidly broken down to acetate by the process of β-oxidation. Previously it was assumed that persistent long-chain

fatty acids would be formed by the breakdown of vegoil which would then be dissolved into the culture to provide a slow continuous source of hydrogen. The lack of such acids implies that the dechlorination zone in a field application may not extend very far from the vegoil itself.

(4) The three types of vegoil (Palm Kernel Oil, Crude Soybean Oil and Refined Bleached and Deodorized Soybean Oil) basically stimulated similar dechlorination, fermentation and methanogenic behaviors.

(5) Comparison of vegoil- and HRC®-fed bottles demonstrated that HRC® stimulated faster dechlorination than vegoil. Hydrogen levels were also consistently lower than those of vegoil (ranging from 1×10^{-7} atm to 1×10^{-5} atm). Based on experimental results in this study, HRC® seems to perform better than vegoil. However, one still needs to judge the cost-effectiveness of HRC® against that of vegoil and its secondary donor, biomass, in order to know which is the more practical method.

(6) Vegoil bottles unamended with yeast extract and vitamins performed at least as well as vegoil bottles amended with yeast extract and vitamins. Completeness of dechlorination (to VC

and ETH) was generally the same for both kinds of systems. This suggests that vegoil possesses (or causes fermentation of) some of the nutrients contained in our yeast extract and vitamin solutions. However, somewhat faster hydrogen production, resulting in faster dechlorination rates, was observed in nutrient amended cultures. This seems to suggest that yeast extract and vitamin B_{12} may play a role in catalyzing the fermentation reaction, although further research would be needed to confirm this.

(7) Biomass accumulation was significant in the bottles. Both experimental observations and bioenergetic calculations showed that the yield due to vegoil utilization was 9 mg biomass/100 mg vegoil. It was suspected that the accumulation of such a large quantity of biomass probably provided sufficient endogenous decay to drive dechlorination. During the long periods of starvation where vegoil was already depleted and endogenous decay was occuring, successive PCE peaks were dechlorinated while hydrogen levels were kept under 1×10^{-5} atm. Methanogenesis was virtually non-existent. Based on these experimental observations, biomass seems to show some

promise as a potential electron donor. However the low-yield of methanogenic cultures may be a drawback since it would make the growing up of cultures to the desired mass an inefficient process.

(8) Comparison of refined bleached and deodorized soybean oil and crude soybean showed similar dechlorination performance. This at least suggests that inhibitory compounds are not found in the non-triacyglycerol constituents of natural vegoil.

CHAPTER SEVEN

ENGINEERING SIGNIFICANCE

Earlier in Chapter Two, methods of applying vegoil technology in the field were discussed. Results from these microcosm studies suggest that some application methods may be impractical, while other, new methods of application may be proposed.

7.A. Significant Observations in Using Vegoil as Fermentable Substrate for Reductive Dechlorination

Two important observations of vegoil were discovered during the lab studies:

1. Vegoil has the potential to ferment rapidly under the given laboratory conditions. However, it is yet unknown whether this will happen at lower temperatures and in much-lower-agitated environments.

2. Persistent long-chain volatile fatty acids that could act as long-term sources of soluble slow-fermenting substrate were not observed in any of the bottles.

The impact of the above-mentioned observations on field application of vegoil are explained in the following section. Recommended modifications to the original engineering systems are also described.

7.B. Impact of Experimental Observations on Field Application of Vegoil Technology

7.B.1. Rapid Fermentation of Vegoil

Originally, it was thought that the slow dissolution of vegoil would allow a single, low cost, injection to provide sufficient carbon to drive reductive dechlorination for a contaminated site for many years. This was because vegoil was assumed to react slowly with fermenters since its low solubility limits its availability to the fermenters. However, experimental conditions in this research, i.e. relatively high temperature and strong

agitation, revealed vegoil is capable of being rapidly fermented, despite its low-solubility.

The possibility of solubility failing to regulate substrate availability under certain physical conditions certainly shows vegoil technology's limitation in field application, though agitation in the field is expected to be far less than employed with these microcosms. During treatability studies, physical conditions in the field must be carefully factored-in to ensure that slow dissolution of vegoil could co-exist with an adequate rate of dechlorination.

In order to remedy the problem of rapid fermentation of vegoil, it is suggested that the chemical or physical structure of vegoil be altered so that it becomes less-readily dissolved. One way is to solidify vegoil under field conditions (such as hydrogenating unsaturated bonds within the carbon skeleton of the fatty acids) or simply to choose a solid vegoil such as Palm Kernel Oil. The solid vegoil can be used as per solid substrates in bio-barriers (see Chapter Two). The advantage of this method is that large amounts of substrate can be added at one time without constant injection and removal. One of the drawbacks is that extensive excavation would be needed to emplace the substrate.

7.B.2. Lack of Persistent Volatile Fatty Acids

Regardless of whether vegoil is rapidly or slowly fermented, the lack of appearance of medium-molecular-weight fatty acids suggests that the benefit of vegoil will not travel far from the oil itself. In the field, this would mean that the dechlorination zone would be limited to the vegoil/water interface and would not extend into the contaminant plume.

If a broader area contact is desired, means must be sought to spread the oil better. Vegoil may be emulsified with water either by using an emulsifier such as lecithin or by applying ultrasound to break up the vegoil. The resulting emulsion may then be injected into the contaminant plume as per dissolved substrates with the donor:PCE ratio controlled by adjusting the injected amount. Essentially the surface area for dissolution of a large LNAPL globule is far lower than that of the equivalent mass of an emulsified vegoil. Such "spreading" techniques will, however, likely lead to much more rapid fermentation of the vegoil as they aid in its solubilization rate. Thus, some of the purported benefits of vegoil – i.e., slow fermentation – may be lost if one seeks to distribute it better. In other words, emulsified vegoil may perform no better than other soluble donors. However, this is mere speculation since no detailed research has been done regarding this. In any event, however, vegoil is likely far less

expensive than is USP-grade lactate. Thus in-terms of cost-effectiveness, vegoil could still possibly be a viable competitor against conventional soluble substrates.

Another method may be to modify the physical structure of the vegoil layer to increase contact surface. An example would be to solidify vegoil into granular form and use it to create a porous structure for contaminated plume to flow through. A trench perpendicular to the groundwater flow could be backfilled with sand particles coated with solid-phase vegoil]. The relatively permeable sand barrier would channelgroundwater flow through it, and its porous structure would increase contact area between the vegoil treatment zone and the contaminant plume (See Figure 7.1). If the benefit of slow-fermentation is lost upon conversion of vegoil to forms that are better distributed, then this implies that the best use will, indeed, be in barriers. The disadvantage is the high costs involved in excavating the trench.

A third method would involve vegoil in its natural LNAPL form. Vegoil could be injected deeper into the groundwater and allowed to surface slowly to the water table. Contact area and time would be increased as a result. (See Figure 7.2). However, it is not known how readily vegoil would surface at all through tight soils.

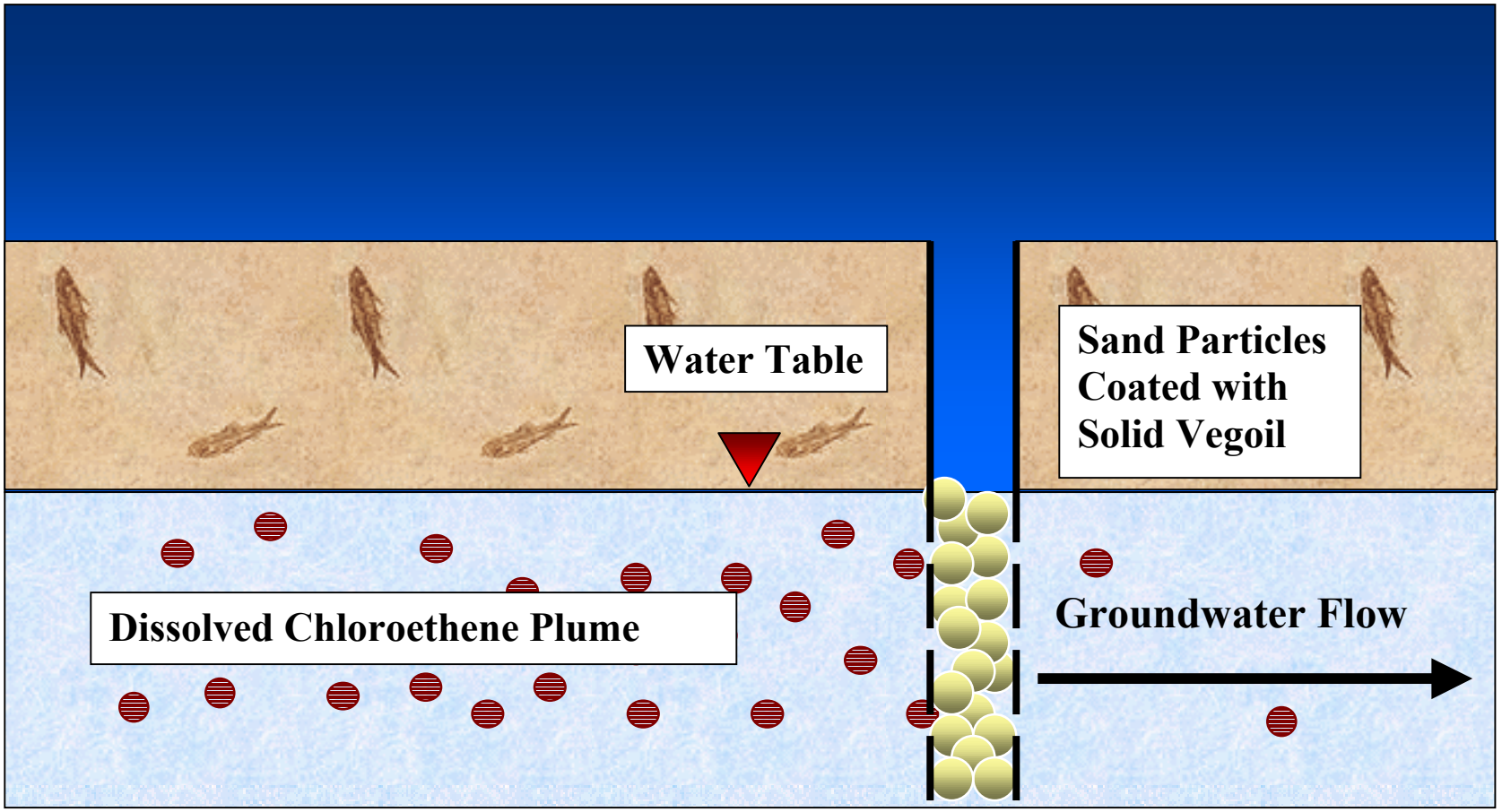

Figure 7.1 Use of Solid Vegoil in Bio-Barrier Role

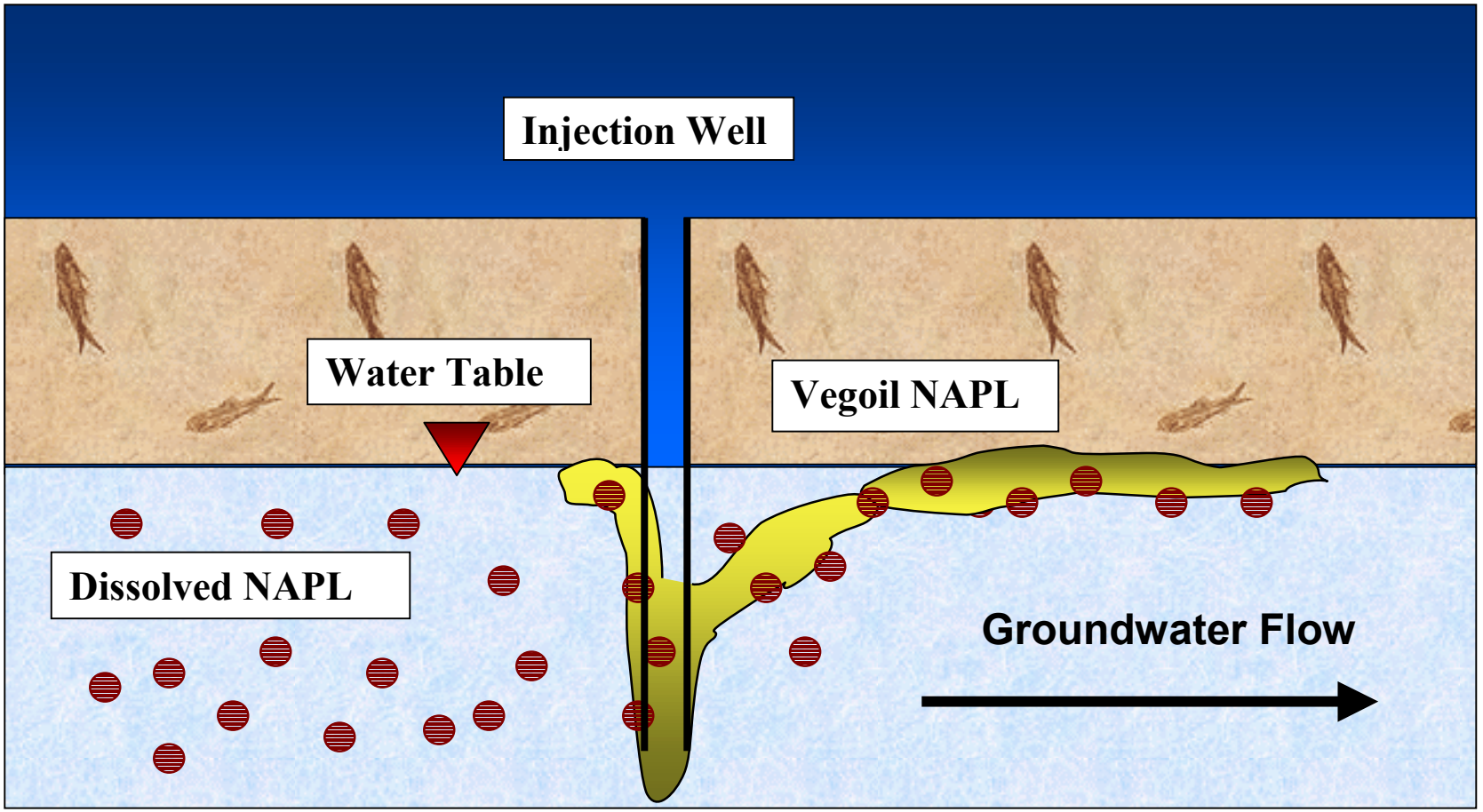

Figure 7.2 Deeper Injection of Vegoil NAPL for the Purpose of Creating Larger Area of Contact.

7.B.3. Potential Use of Biomass as Persistent Secondary Donor

Experimental results show to some degree the secondary benefit of vegoil's causing growth of biomass. Endogenous decay of biomass apparently served as an exemplary donor for reductive dechlorination in our study. However, one needs to be concerned about aquifer-fouling (either directly from biomass, or from gas-binding caused by methane bubbles), potential hazardous levels of methane generation, and the fact that biomass tends not to be very mobile. Thus, the biomass formed in the vicinity of the vegoil may not provide donor effect distant from the source of its growth. However, this is conjecture and physical models or actual site investigations would be needed in order to confirm this.

7.C. Other applications of Vegoil in the Field

Vegoil's non-polar properties could make it excellent for capturing chloroethenes when they first leak out of their storage tanks. Partitioning of chloroethenes into the vegoil layer could possibly prevent further migration into the groundwater and accumulation on the bedrock/aquitard.

Since vegoil is an LNAPL, the resulting chloroethene/vegoil mixture could potentially remain above the groundwater table (provided that the final density of the mixture is less than water). Source removal of the contaminant would be much easier since the leaked chloroethenes no longer migrate through the groundwater and accumulate as DNAPL. It would also be possible that some reductive dechlorination of the partitioned chloroethenes could take place around the vegoil layer. (See Figure 7.3)

7.D. Suggestions for Further Research

Some of the projections made in the previous sections require further study in order to verify their feasibility. In this section, several important areas of investigation for exploring the possible applications of vegoil are listed:

1. Is vegoil fermented slowly at temperatures more reflective of average field conditions? For example, at 15^{o}C and minimal agitation?

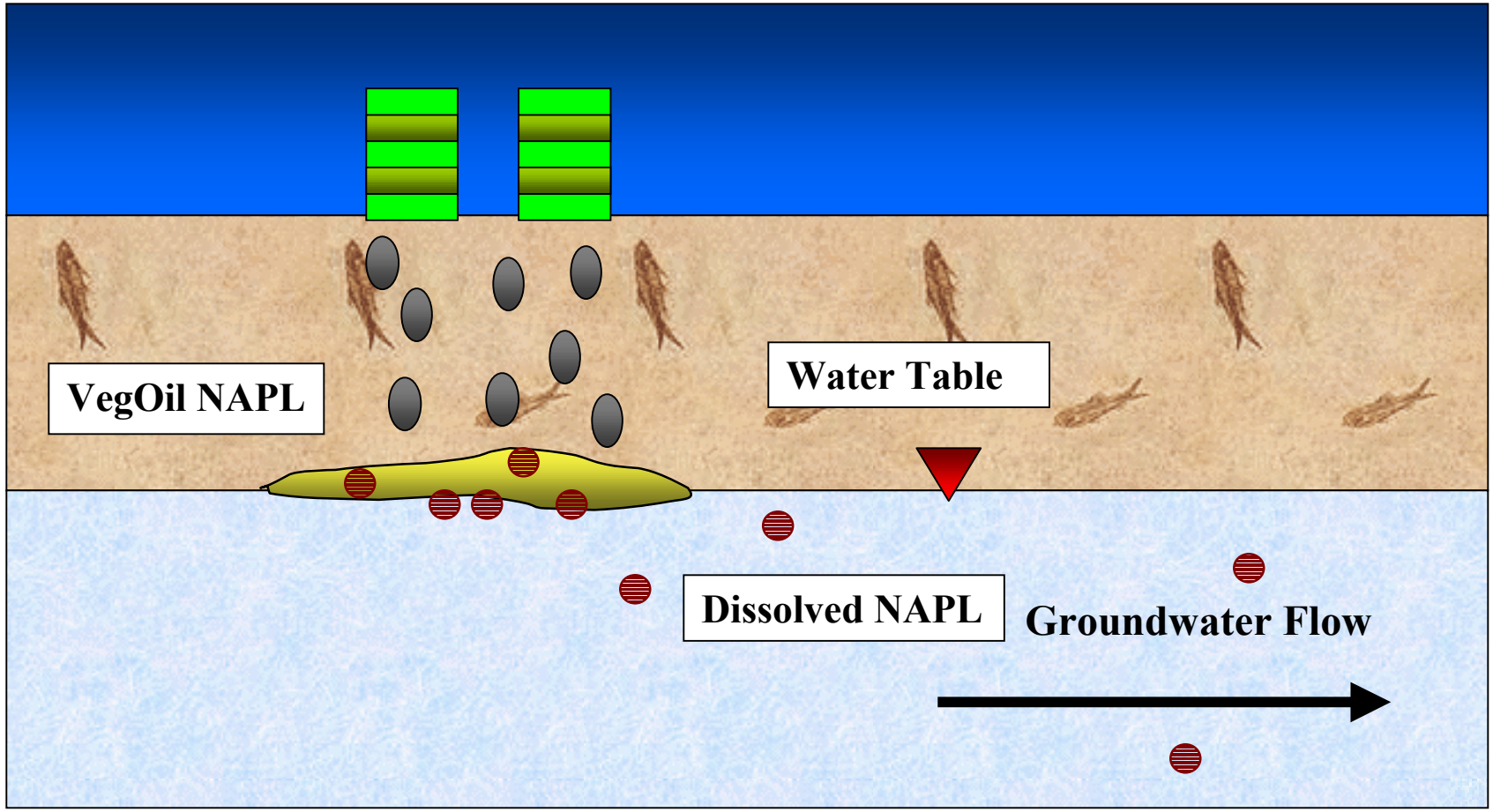

Figure 7.3 Use of Vegoil LNAPL as a Barrier against Downward Migration of Chlorinated DNAPL

2. Can emulsified vegoil perform just as well as well-studied soluble substrates such as lactate, butyrate and propionate? What would be its comparative cost-effectiveness?
3. Can biomass be effective in extending the dechlorination zone?
4. Can vegoil-coated sand barriers act as effective bio-barriers?

To answer questions 1 and 2, microcosm studies similar to this study could be carried out. For instance, to evaluate vegoil's performance under average physical field conditions, similarly amended bottles can be incubated at lower temperatures and slower agitation rates. Furthermore, to reflect indigenous microbial populations, soil slurries from successfully treated sites can be used in place of the low PCE/butyrate culture applied in this study. Apart from serum bottle studies, soil-bed or soil-column studies can be applied to better reflect actual field conditions.

As for evaluating the cost-effectiveness of emulsified vegoil, the protocol used for operating microcosms with soluble substrates (developed by Fennell [*20*]) can be applied. This protocol would necessitate feeding and wasting at much narrower time-intervals designed to reflect the operation of an *in-situ* injection/recovery treatment system.

To answer questions 3 and 4, it is suggested that column studies be carried out. For relatively passive systems such as bio-barriers or monitored natural attenuation, soil column tests could best reflect the conditions that are encountered by the systems as well as to test their treatment efficiency.

Because methanogenesis was such a predominant activity in the vegoil bottles in this study, it is suspected that the gas-clogging caused by it could adversely affect dechlorination in actual aquifers. This is particularly severe in cases of bio-barriers, because the low permeability in the affected soil could divert the contaminated plume away from the treatment zone. Soil columns fitted with piezometers along it could be used to quantify headlosses caused by possible gas-clogging.

The extension of the dechlorination zone caused by the migration of biomass could also be investigated using soil column tests. Sampling ports situated along the column could be use to detect areas where dechlorination reactions are taking place. Comparison of the total dechlorinating column length with the vegoil portion could possibly be used to infer the extent of biomass-driven dechlorination.

APPENDIX A

TIME COURSE PROFILES FROM SERUM BOTTLE STUDIES

The various time-course profiles (chloroethenes, methane and hydrogen, and the vegoil breakdown products) from the serum bottles used in this study are shown in this section. With the exception of those displayed in chapter 4, all triplicates of vegoil, HRC® and unfed control bottles are included in this section.

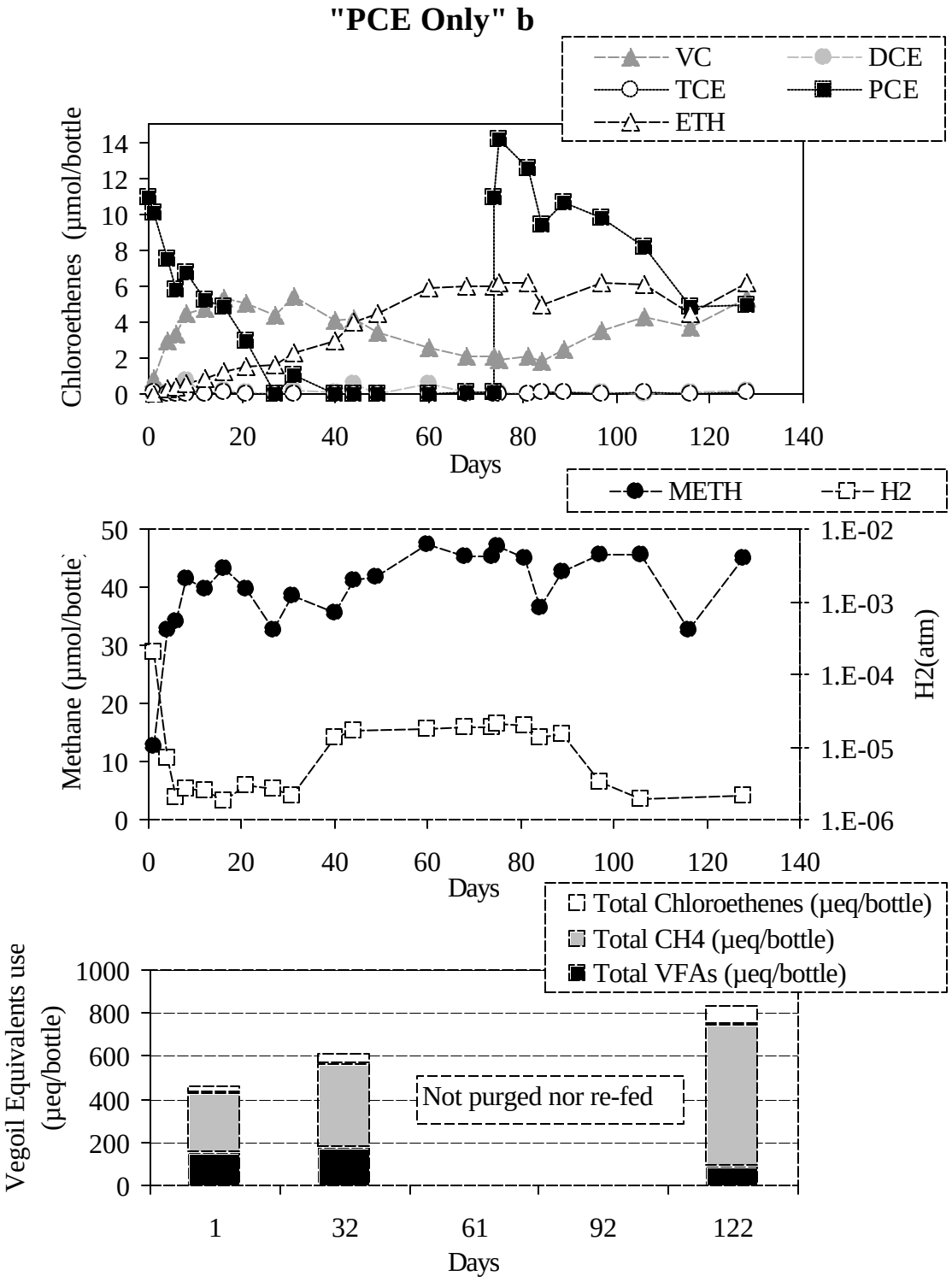

Figure A.1. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil (without Yeast Extract and Vitamins) ; Triplicate b.

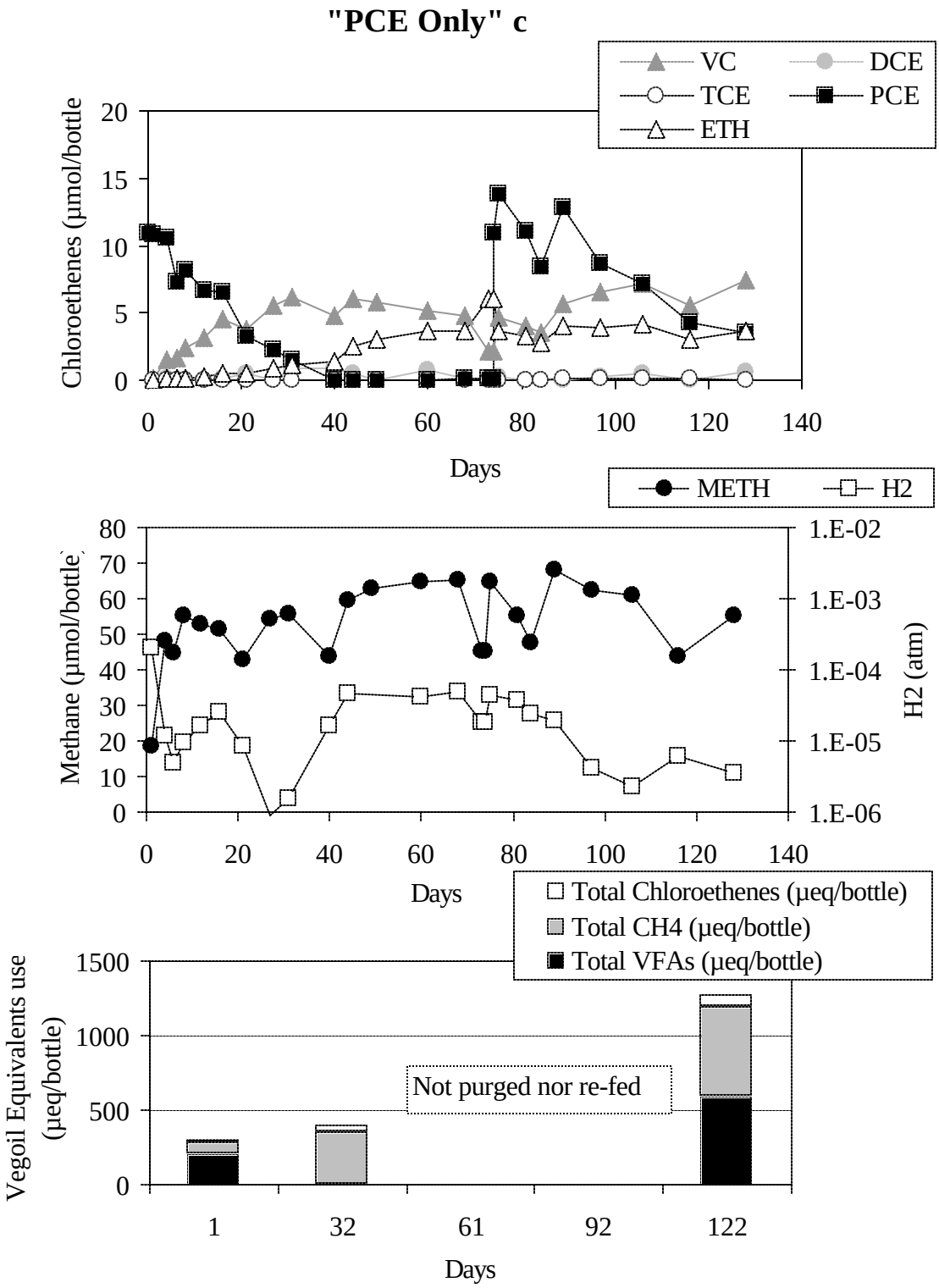

Figure A.2. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil (without Yeast Extract and Vitamins); Triplicate c.

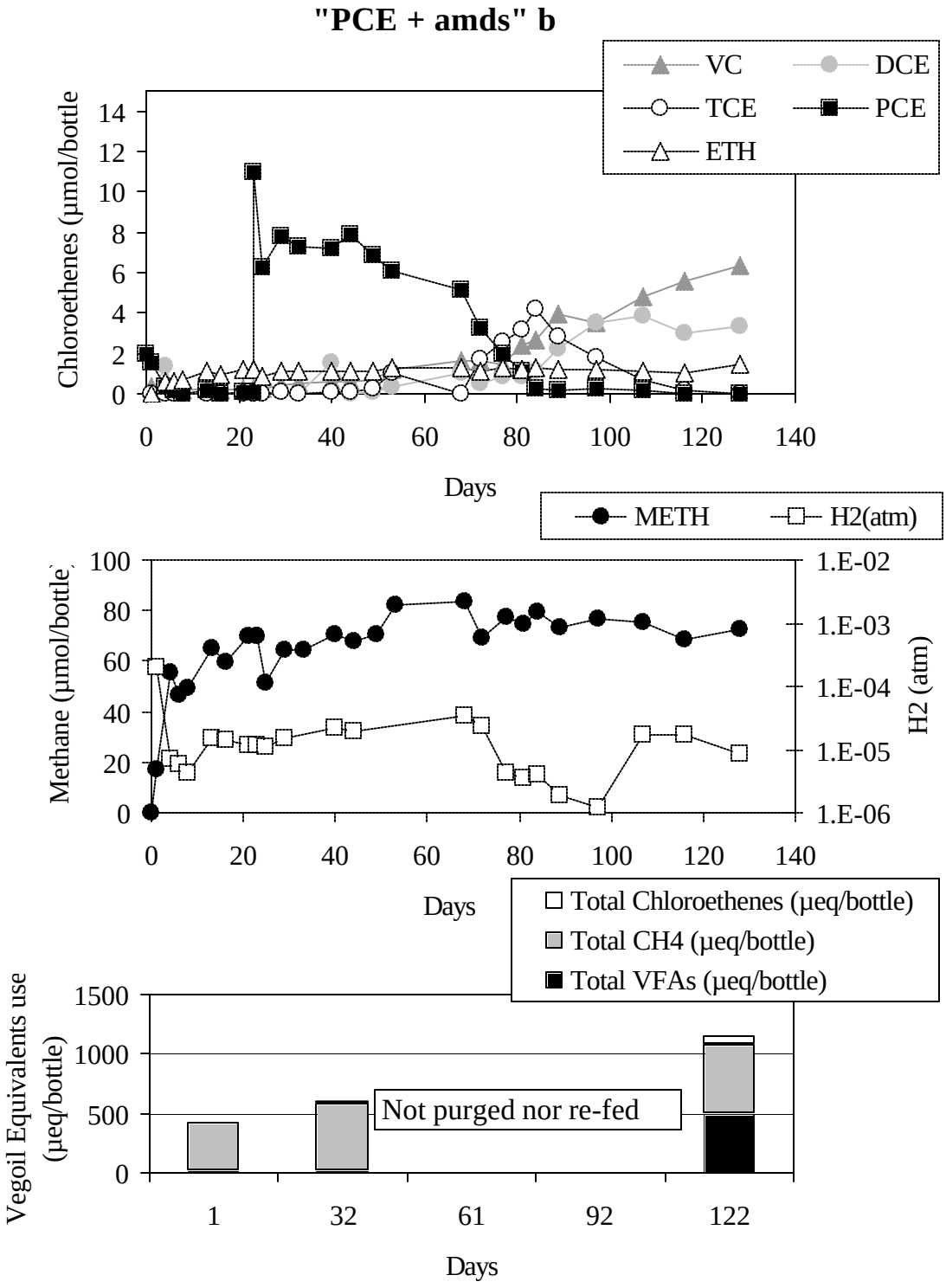

Figure A.3. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil + Vitamins + Yeast Extract; Triplicate b

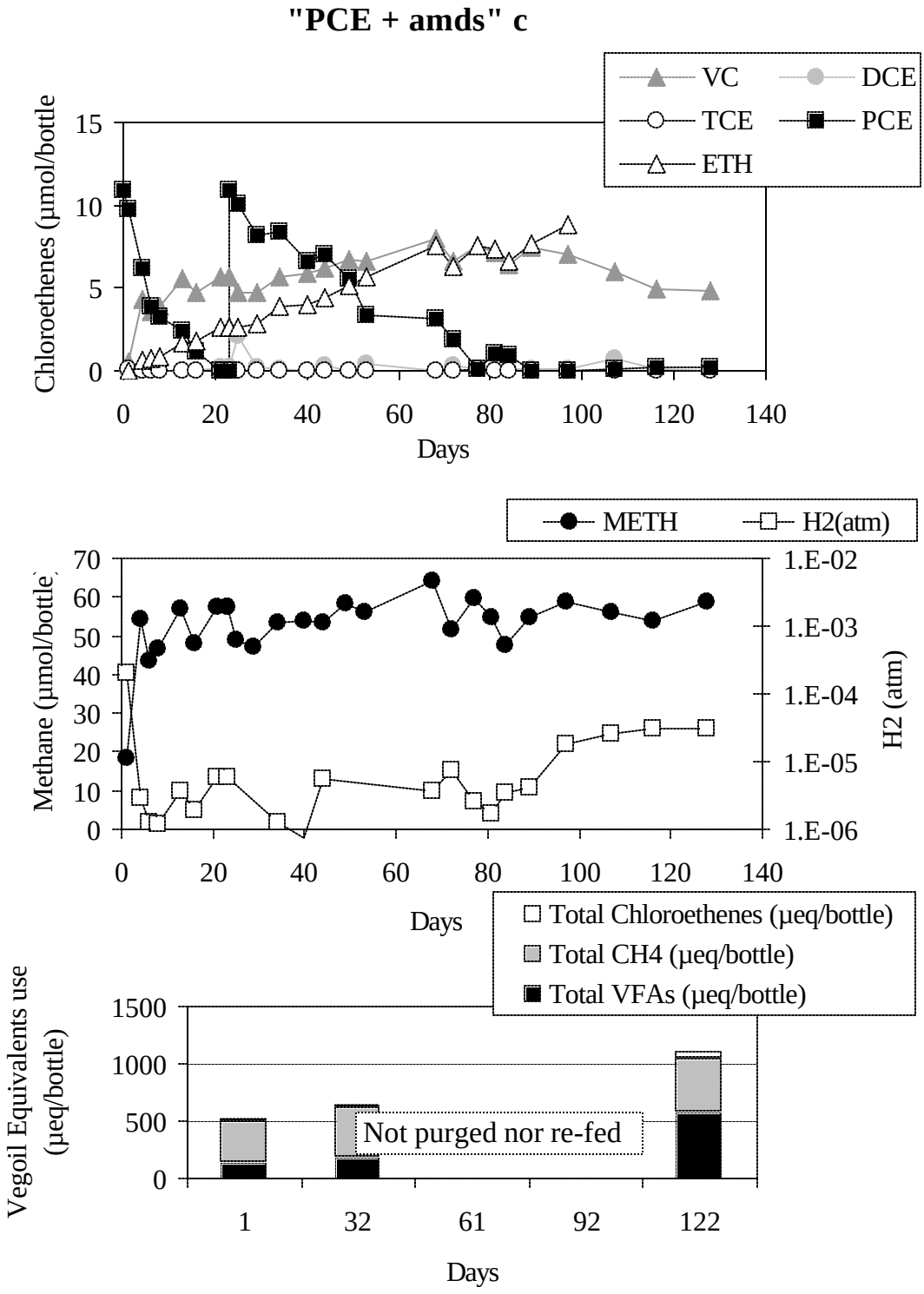

Figure A.4. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for No Vegoil + Vitamins + Yeast Extract; Triplicate c.

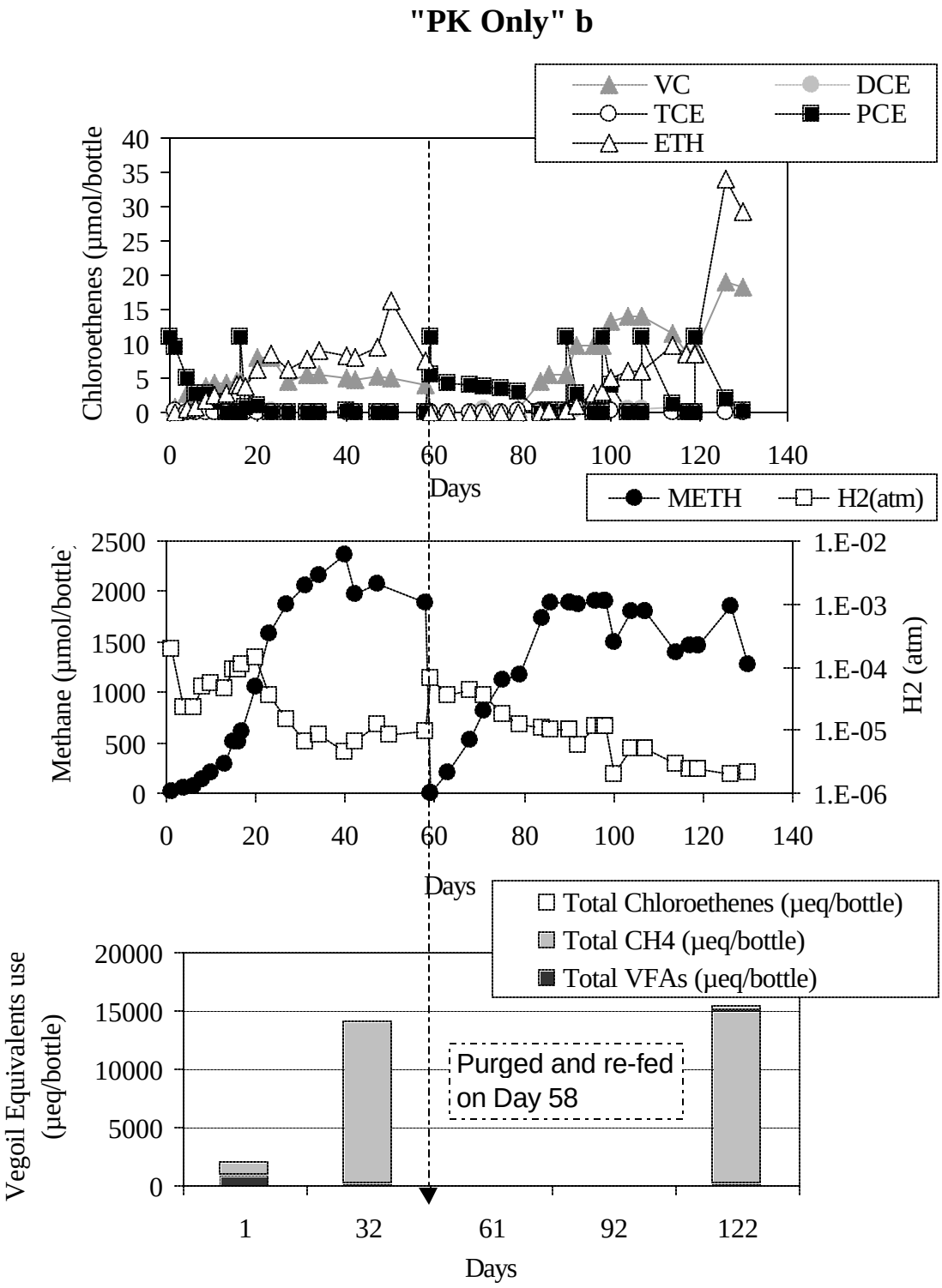

Figure A.5. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil (without Yeast Extract and Vitamins); Triplicate b.

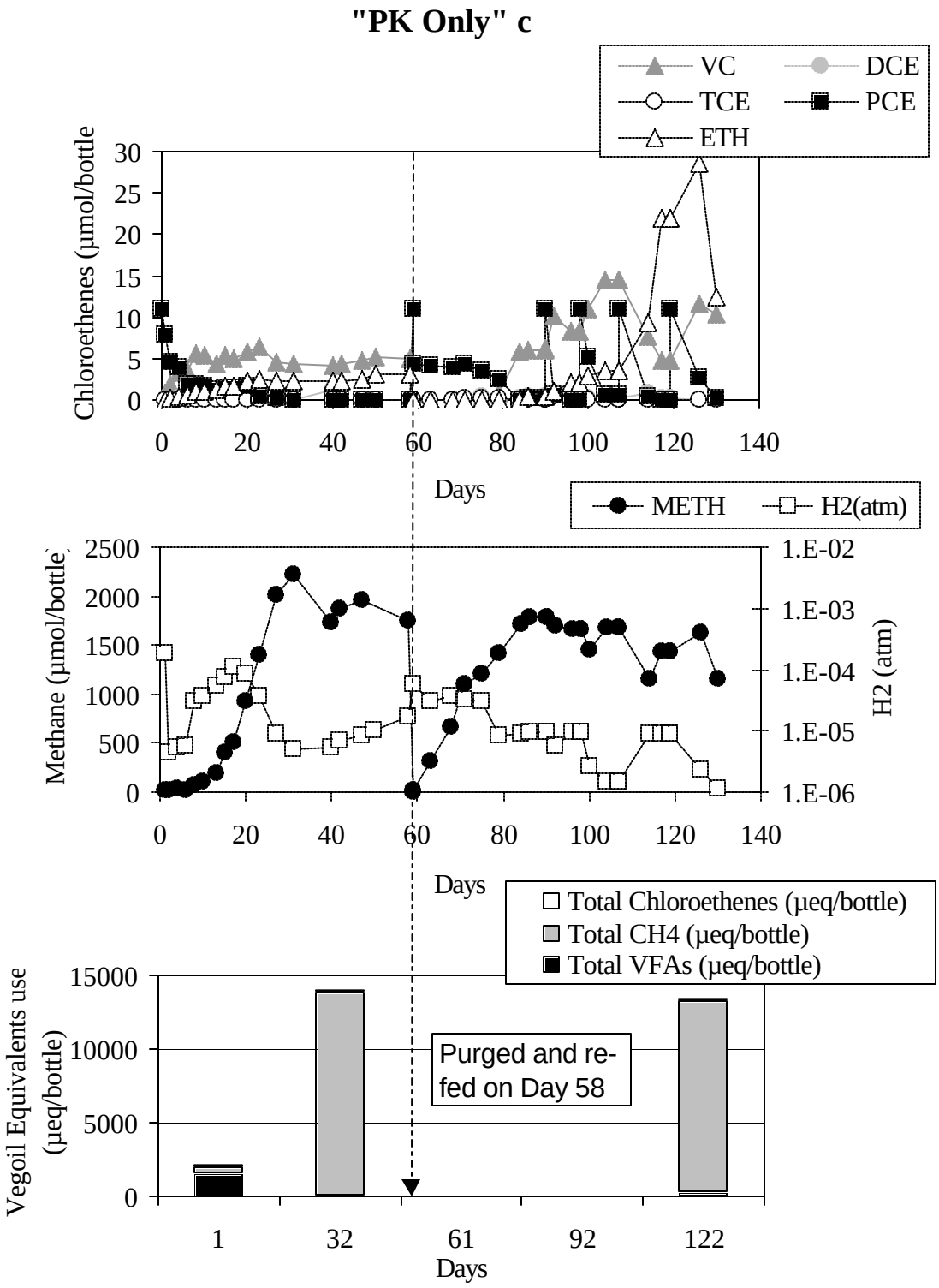

Figure A.6. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil (without Yeast Extract and Vitamins); Triplicate c.

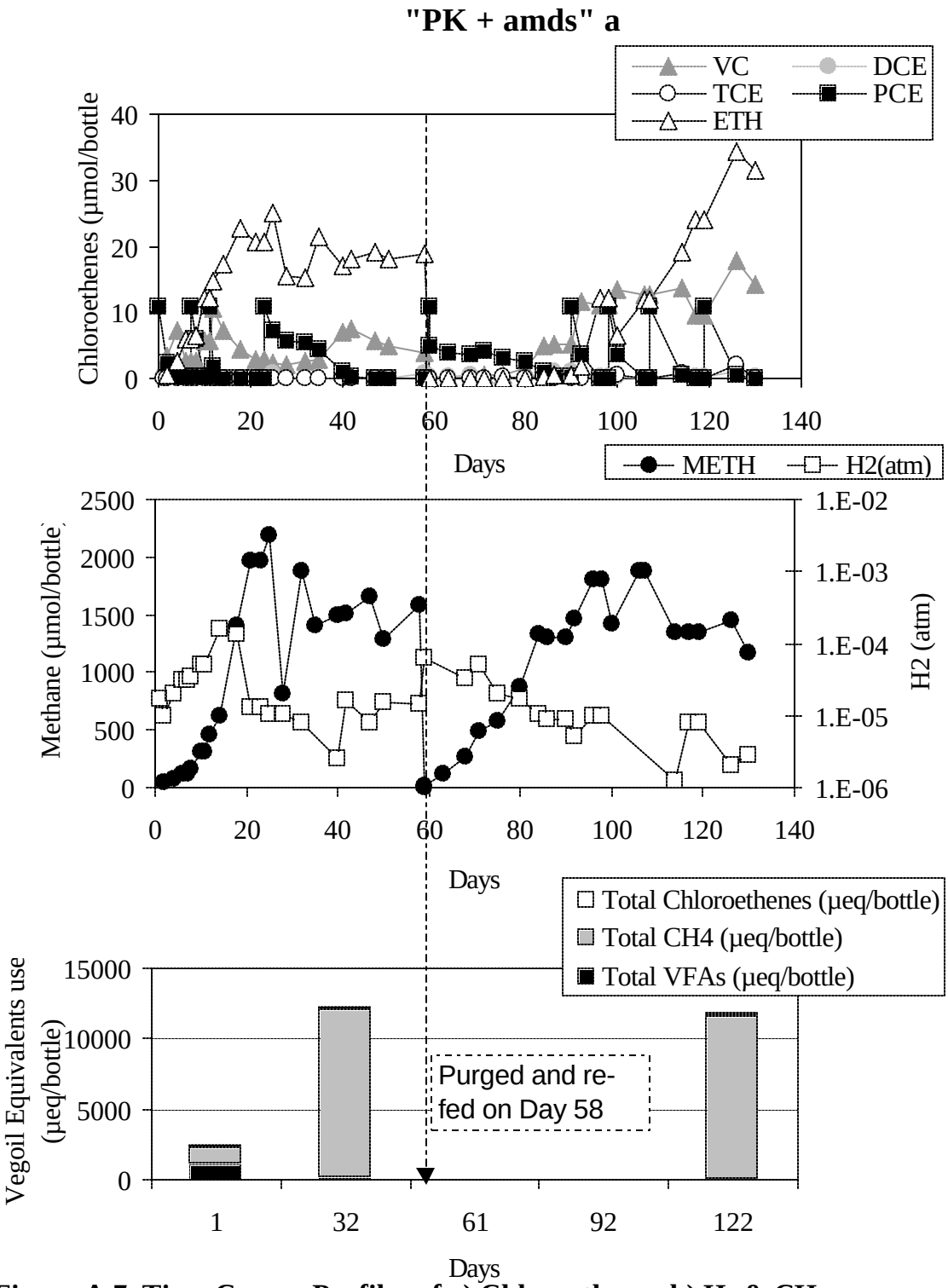

Figure A.7. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil + Yeast Extract + Vitamins; Triplicate a.

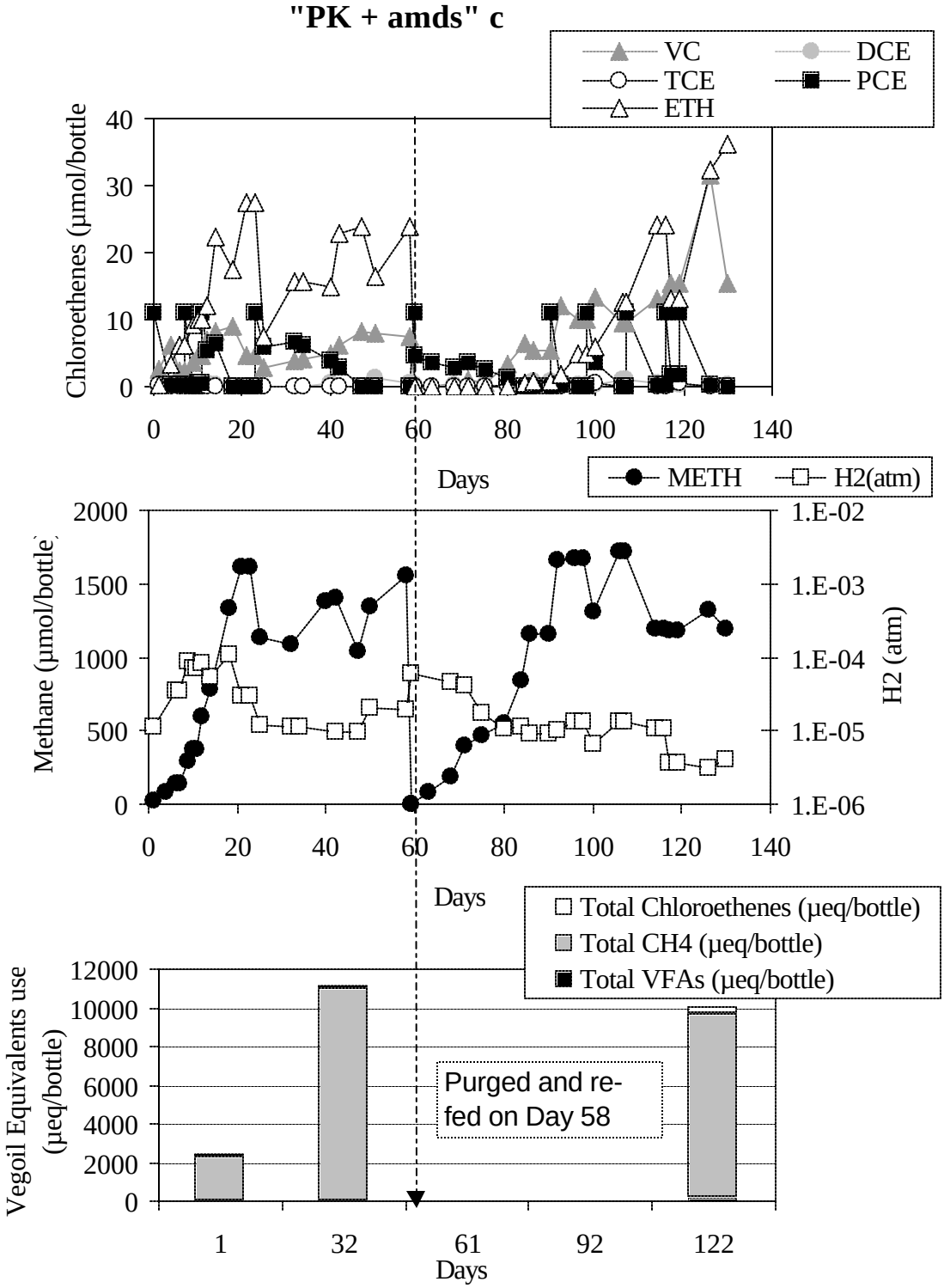

Figure A.8. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Palm Kernel Oil + Yeast Extract + Vitamins; Triplicate c.

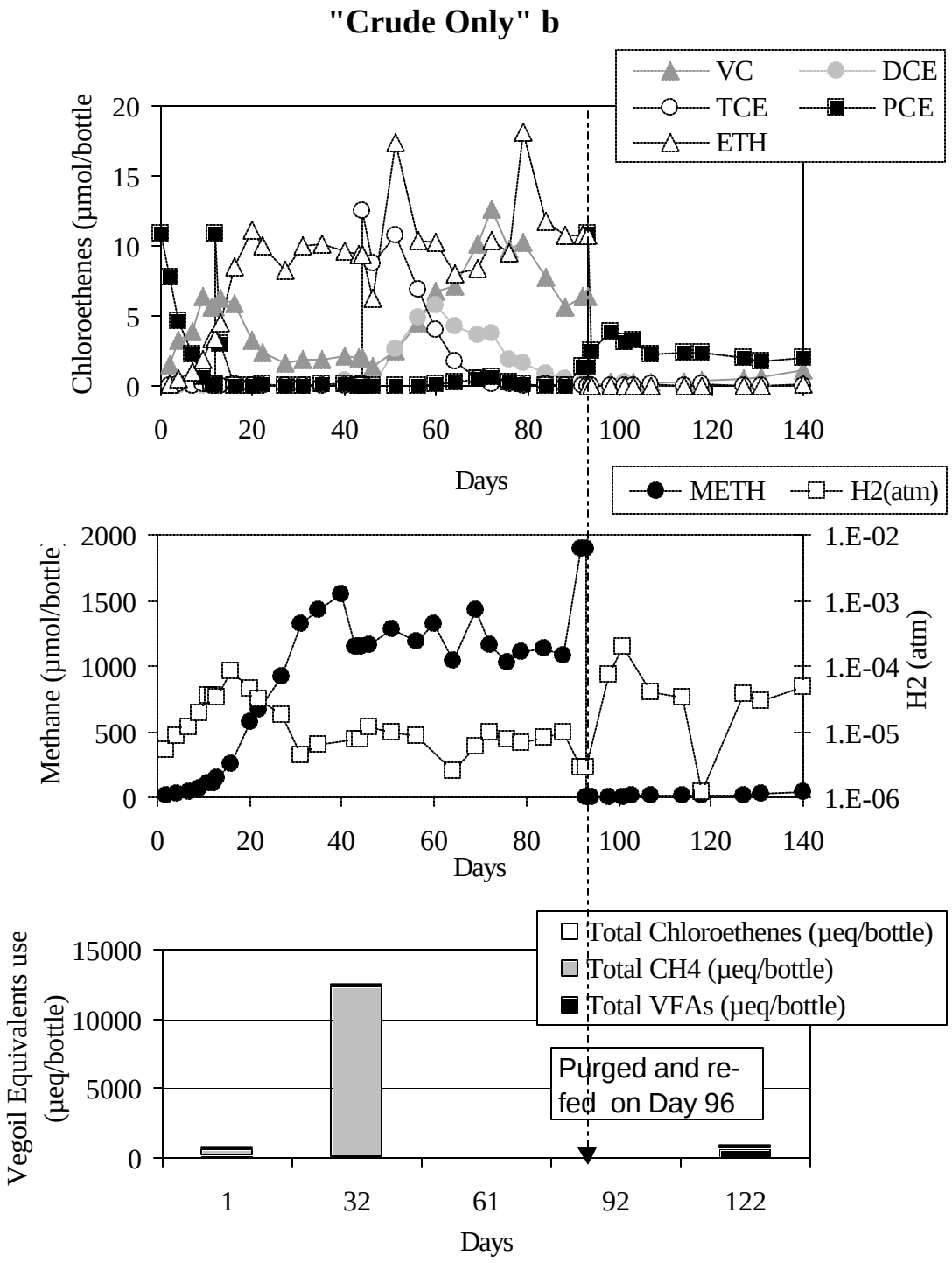

Figure A.9. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil (without Yeast Extract and Vitamins); Triplicate b.

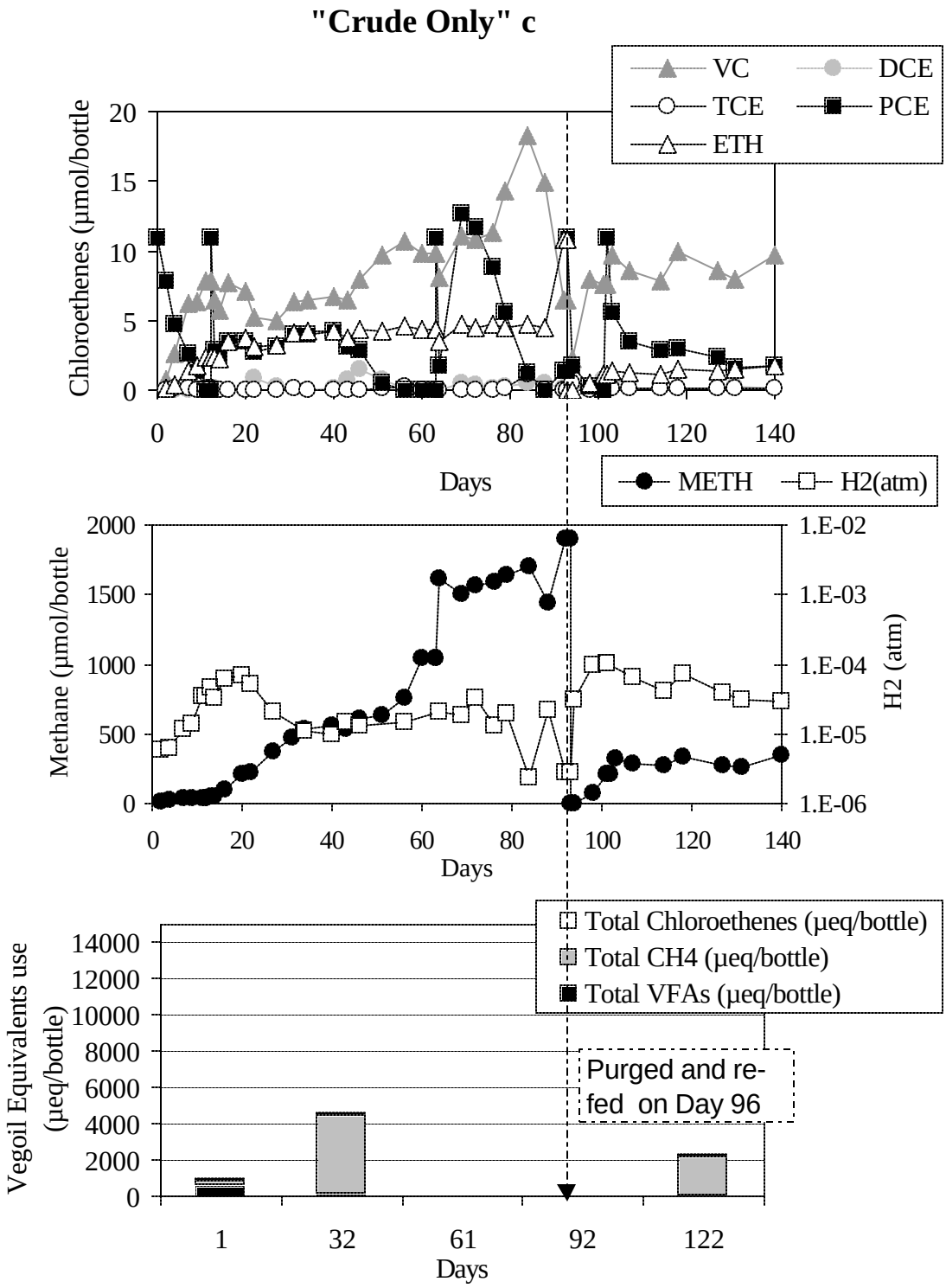

Figure A.10. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil (without Yeast Extract and Vitamins); Triplicate c.

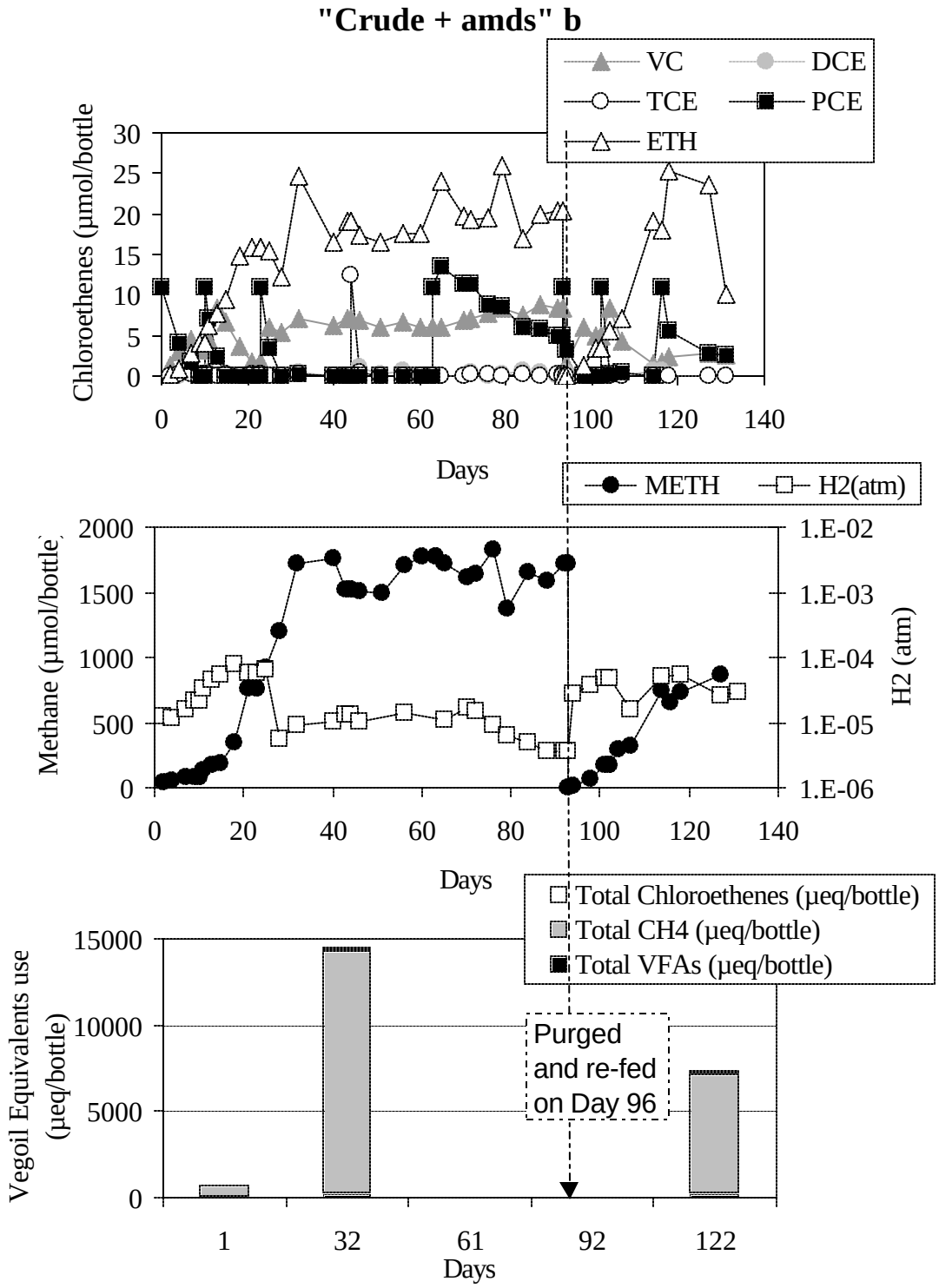

Figure A.11. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil + Yeast Extract + Vitamins; Triplicate b.

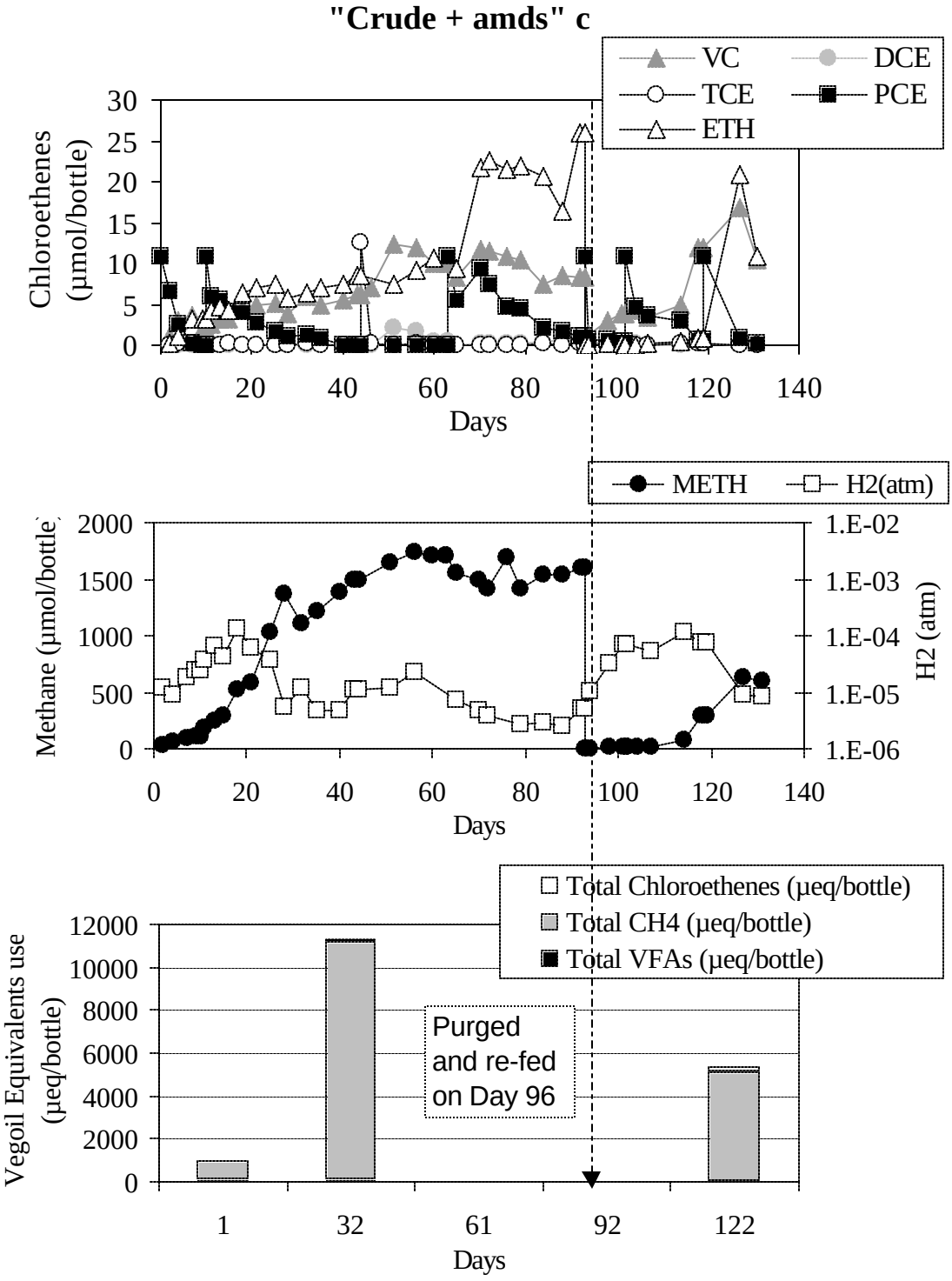

Figure A.12. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Crude Soybean Oil + Yeast Extract + Vitamins; Triplicate c.

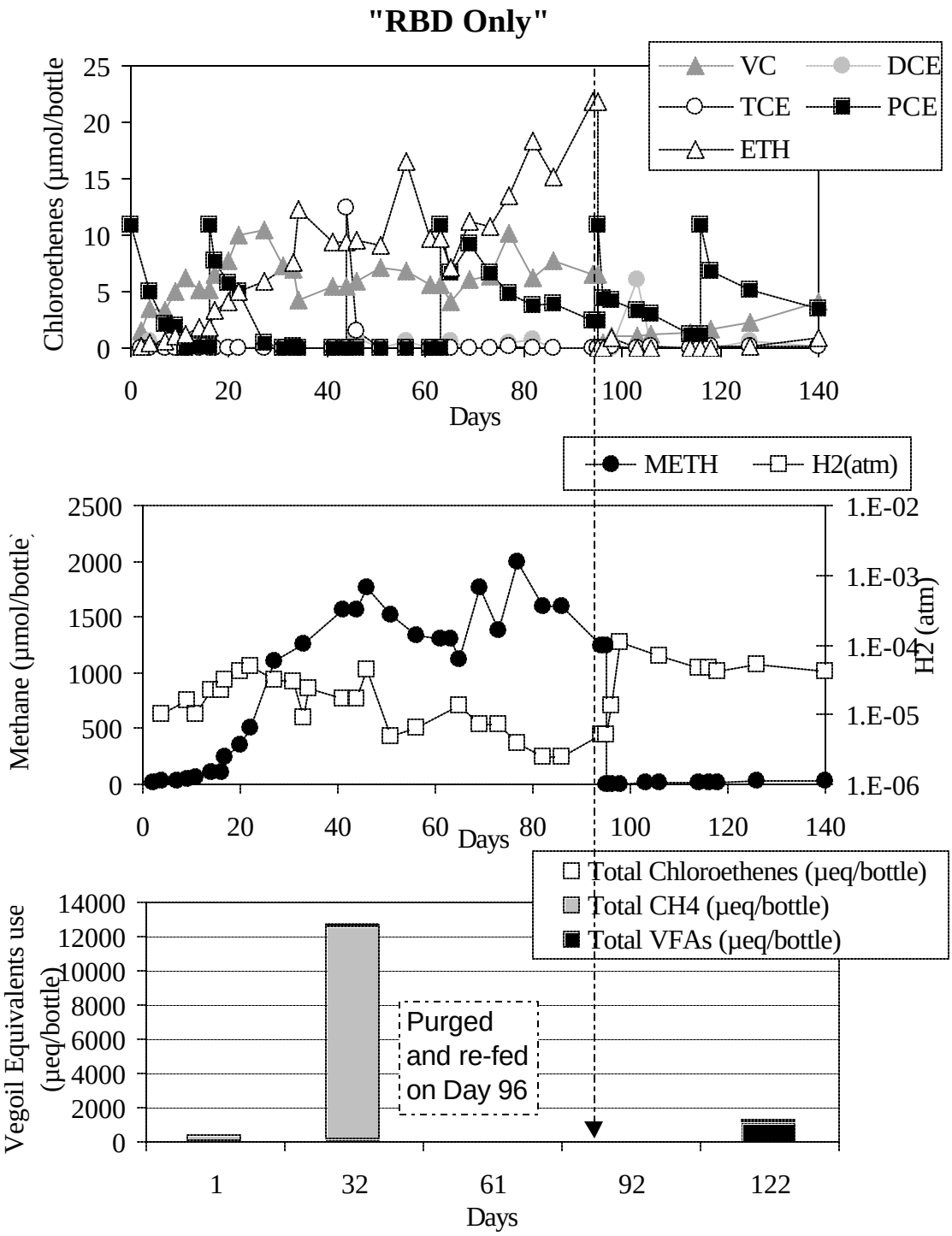

Figure A.13. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Refined Bleached and Deodorized Soybean Oil (without Yeast Extract and Vitamins); Triplicate a.

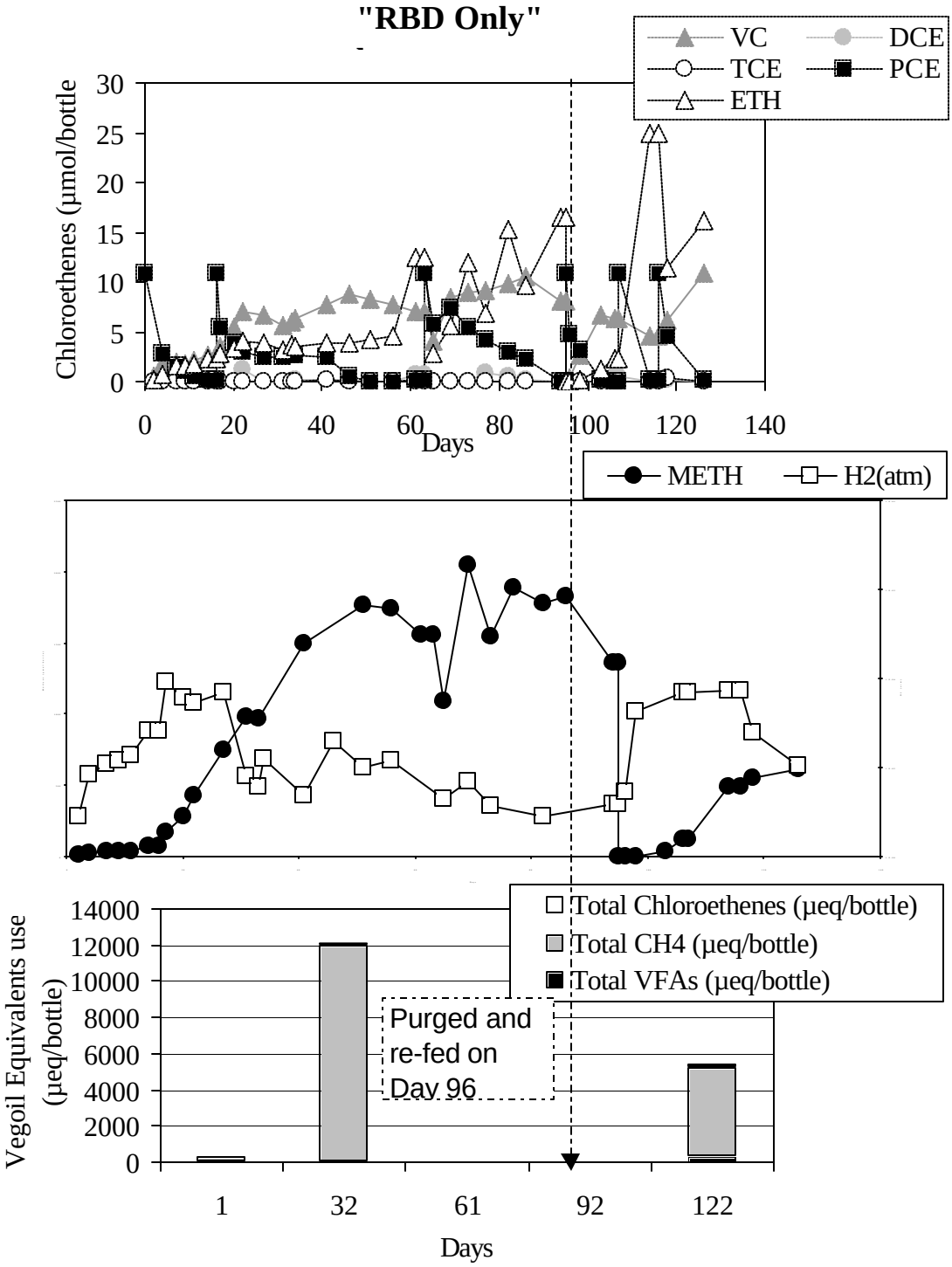

Figure A.14. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Refined Bleached and Deodorized Soybean Oil (without Yeast Extract and Vitamins); Triplicate b.

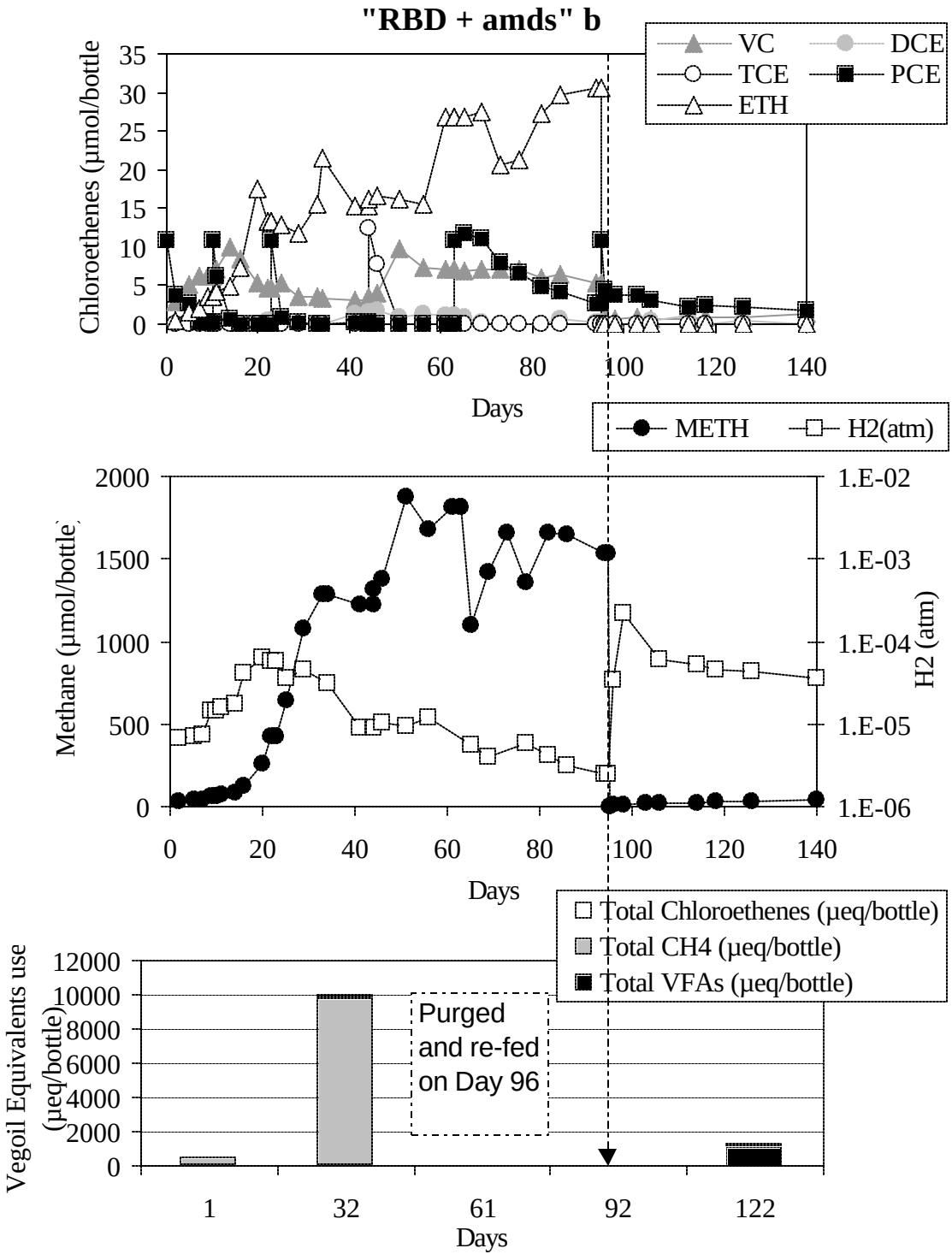

Figure A.15. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for Refined Bleached and Deodorized Soybean Oil + Yeast Extract + Vitamins; Triplicate b.

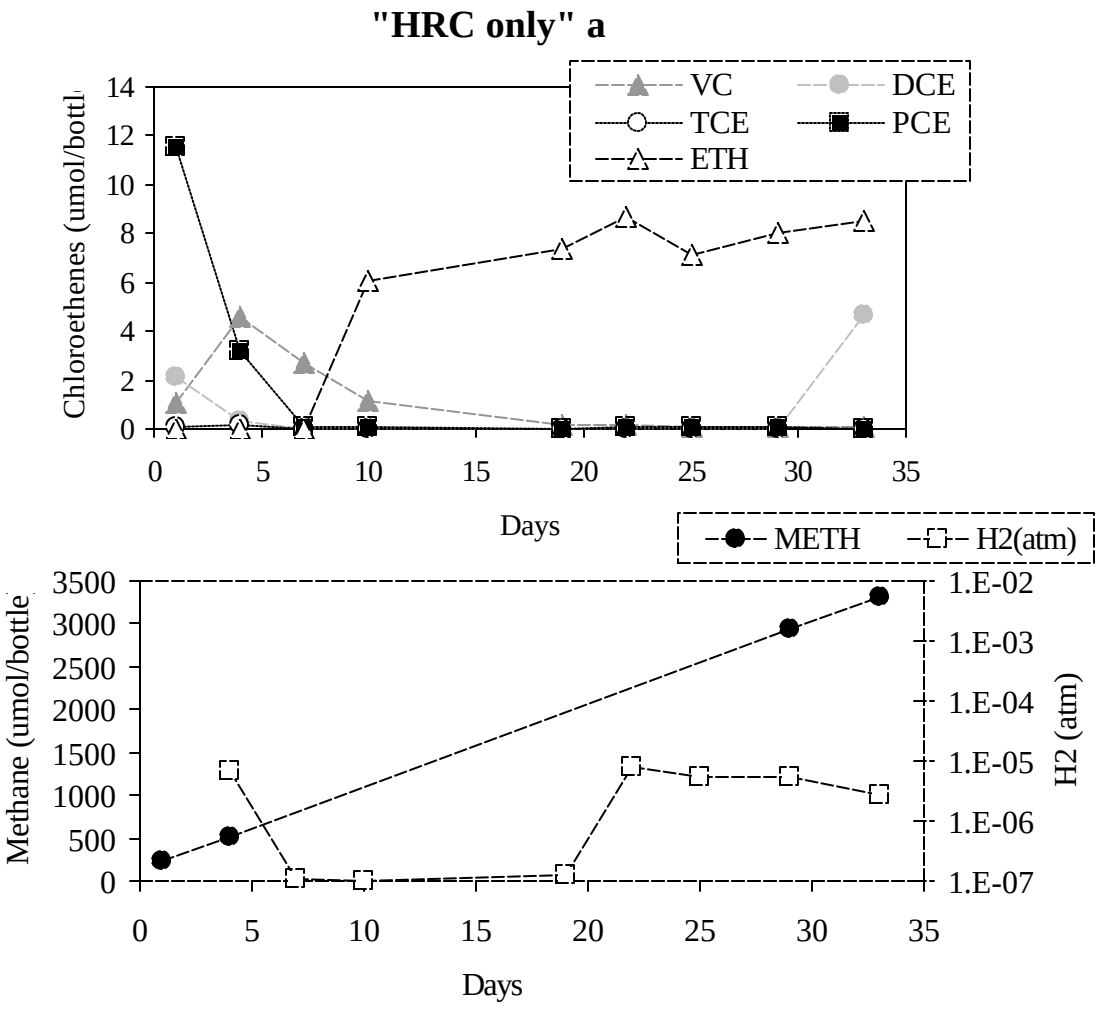

Figure A.16. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) (without Yeast Extract and Vitamins); Triplicate a.

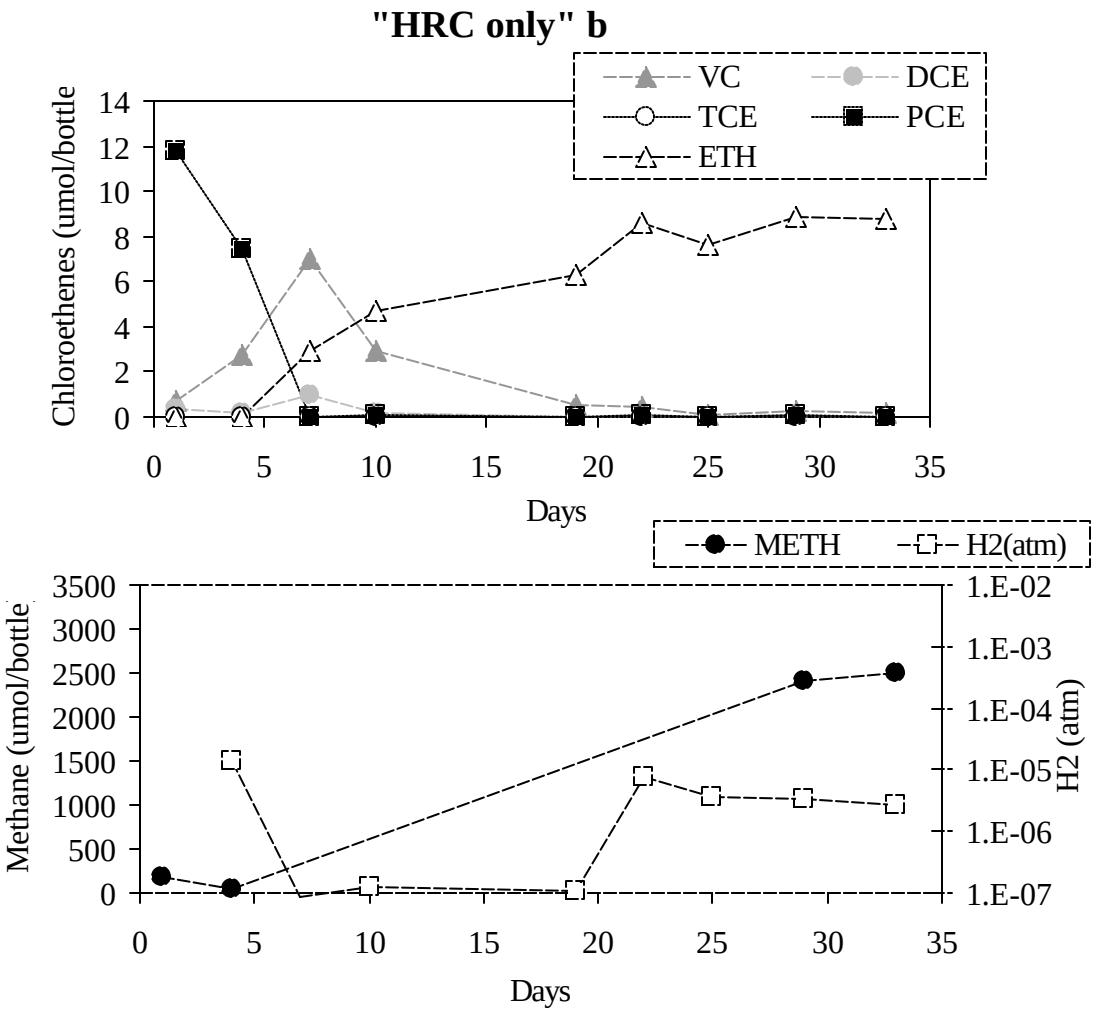

Figure A.17. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) (without Yeast Extract and Vitamins); Triplicate b.

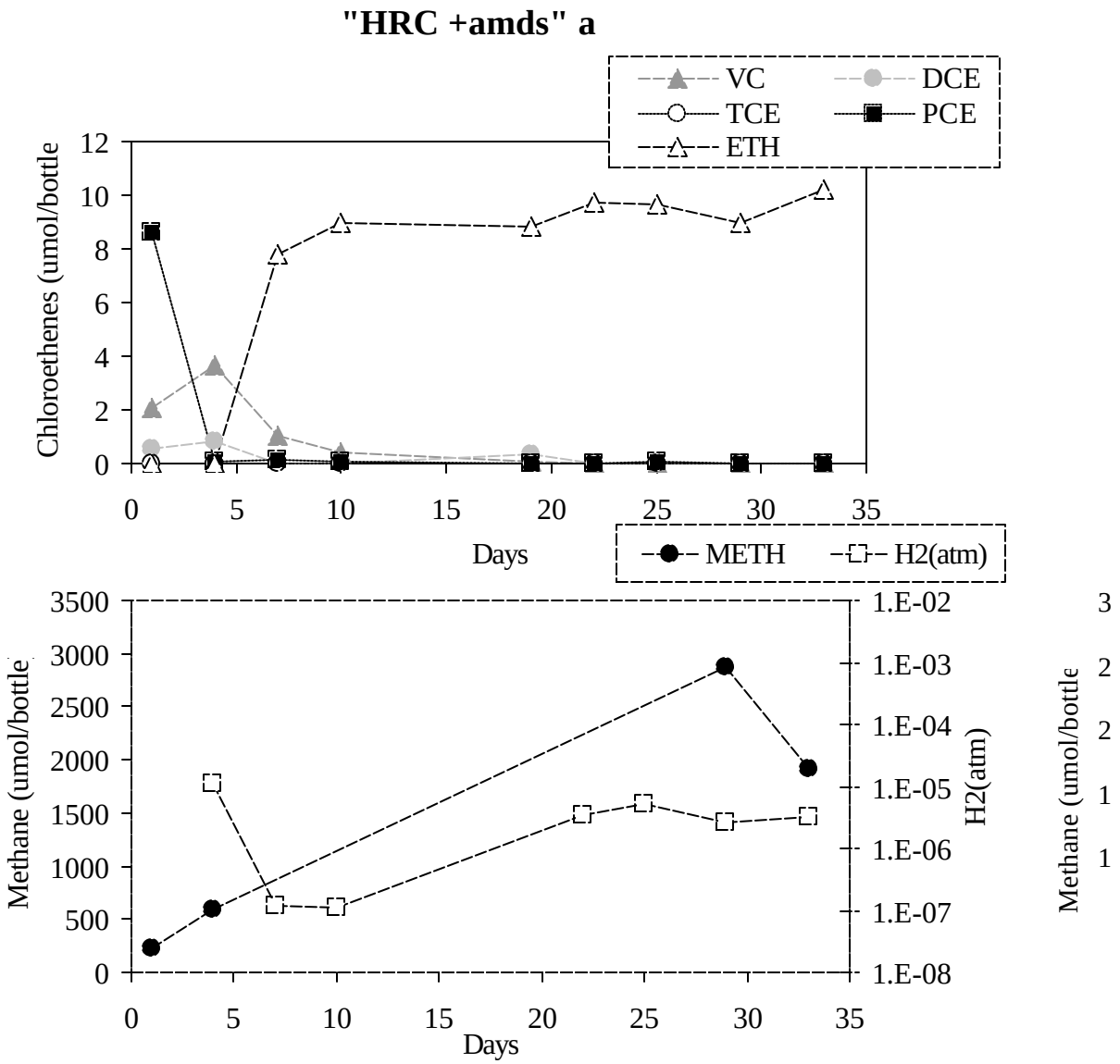

Figure A.18. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) +Yeast Extract + Vitamins; Triplicate a.

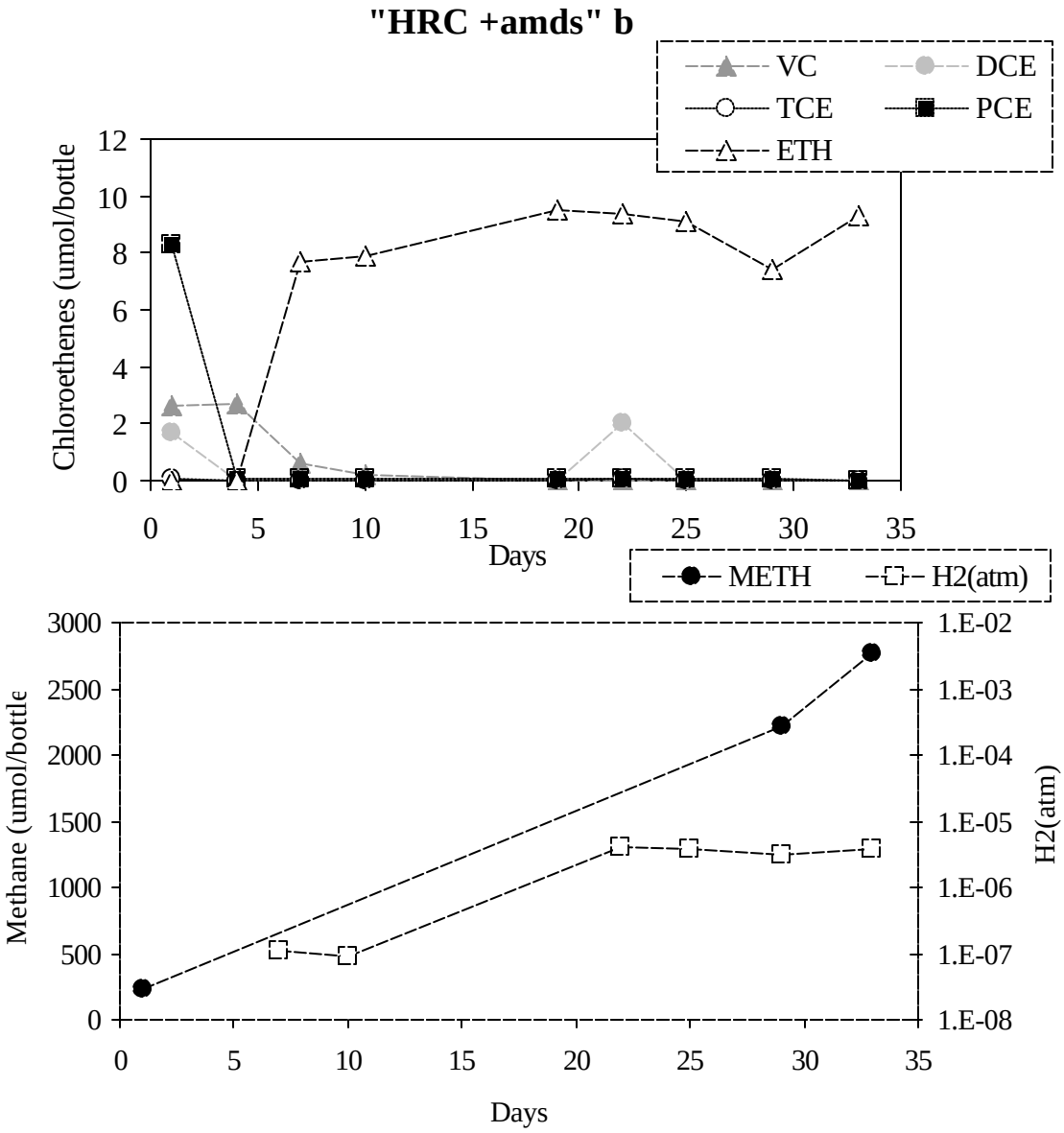

Figure A.19. Time Course Profiles of a) Chloroethenes b) H_2 & CH_4 and c) Vegoil Breakdown Products for HRC(R) + Yeast Extract + Vitamins; Triplicate b.

APPENDIX B

BIOMASS CALCULATIONS

In this section, we attempt to verify measured biomass values with bioenergetic calculations. We first calculate the theoretical yield based on the Gibb's free energies of the oxidation half reactions of vegoil and methane. Then using the theoretical yield and an assumed endogenous decay rate, we will attempt to calculate the theoretical final biomass and compare it with its experimental value.

Appendix B1. Estimating Yield from Vegoil

From Rittman and McCarty [45], the Gibb's free energy for the half-reaction using oil as an electron donor is given as:

$$\Delta G_{f_{e.d.}} = -\,6.6\,kcal/eeq\ for\ vegoil\ (C_8H_{16}O)$$

while the free energy for the half reaction using carbon dioxide as the electron acceptor (since methanogenesis comprised above 90% of donor usage) is given as:

$$\Delta G_{f_{e.a.}} = -5.763\, kcal / eeq \ for\ CO_2\ (methanogenesis)$$

Following the bioenergetic approach described by Rittmann and McCarty [45], the theoretical yield is calculated to be $Y = 0.252 \frac{gXa}{eeq}$.

Appendix B2. Verifying Theoretical Values with Experimental Data

The measured average biomass in the unfed controls ("PCE only" and "PCE + amds") was 7.1 mg/bottle at the end of the 140-day period. The average amount of biomass in the various vegoil-fed bottles at the end of the 140 monitoring period was 15.76 mg biomass/bottle.

For 100 mg of vegoil, the total amount of equivalents (based on oxidation to CO_2) is equal to 35.940 meq. In a batch reactor, the rate of change in biomass is given by:

$$\frac{dX}{dt} = -Y\frac{dS}{dt} - bX$$

where X = cell biomass (mg $C_5H_7O_2N$)/bottle

S = substrate (meq)

Y = theoretical yield of the culture (mg $C_5H_7O_2N$/meq)

b= endogenous decay rate (d^{-1})

t = time (d)

This expression can be split into two parts:

1) The growth due to substrate utilization part, where

$$(\frac{dX}{dt})_{growth} = -Y\frac{dS}{dt}$$

Integrating, we get

$$\Delta X_{growth} = - Y\Delta S$$

2) The endogenous decay part, where

$$(\frac{dX}{dt})_{decay} = -bX$$

Assuming that X remains more or less constant (within a factor of two of the original cell biomass, X_o) throughout the experiment, the expression integrates out to be

$$\Delta X_{decay} = -X_0(1-e^{-bt})$$

Summing the two parts together, we obtain the expression:

$$X - X_0 = -Y\Delta S - X_0(1-e^{-bt})$$

Expanding the equation, we end up with:

$$X = -Y\Delta S + X_0 e^{-bt} \quad (1)$$

For the unfed controls, the first term on the right is zero since no substrate is fed, therefore:

$$X_{unfed} = X_0 e^{-bt}$$

This was measured to be 7.06 mg/bottle at the end of the 140-day monitoring period, therefore

$$X_0 e^{-bt} = X_{unfed} = 7.06 mg/bottle$$

Substituting $X_0 e^{-bt}$=7.06 mg/bottle, Y = 0.252 mg $C_5H_7O_2N$/meq and ΔS = -35.940 meq into equation (1), we have:

$$X = -0.252(-35.940) + 7.08$$

$$X = 16.14 mg/bottle$$

This figure is close to the measured average biomass value of 15.76 mg/bottle for the vegoil-fed bottles. Therefore we can conclude that biomass measurements generally agree with bioenergetic calculations.

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